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Worming to Complete the Insulin/IGF-1 Signaling Cascade: A Dissertation

Srivatsan Padmanabhan
University of Massachusetts Medical School

A Dissertation Presented

By

Srivatsan Padmanabhan

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The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

Heidi Tissenbaum, Ph.D., Thesis Advisor

T. Keith Blackwell, M.D., Ph.D., Member of Committee

Victor Boyartchuk, Ph.D., Member of Committee

Marian Walhout, Ph.D., Member of Committee

Lan Xu, Ph.D., Member of Committee

The signature of the Chair of Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Craig Mello, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduate requirements of the School

Anthony Carruthers, Ph.D.,
Dean of Graduate School of Biomedical Sciences

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Statement of contribution

Figure 12: The RNAi screen to identify phosphatases acting in the insulin signaling cascade was done by me.

Figure 13-16: were done in collaboration with Arnab Mukhopadhyay and Sri Devi Narasimhan.

Figure 17: was done by Sri Devi Narasimhan and Arnab Mukhopadhyay.

Figure 18, 19, 20: Double transgenics were generated by me. Images were done in collaboration with Arnab Mukhopadhyay and Sri Devi Narasimhan.

Figure 21: was done in collaboration with Arnab Mukhopadyay and Sri Devi Narasimhan.

Figure 22, 23: were done by Arnab Mukhopadhyay.

Figure 24: was done by me.

Figure 25: was done by me.

Figure 26, 27: were done by Greg Tesz in the laboratory of Dr.Michael Czech at UMass.

Figure 28: was done by Sri Devi Narasimhan.

Figure 29: Model is taken from the Cell manuscript.

Table 4, 5, 6: were done by me.

Table 7: was done in collaboration with Sri Devi Narasimhan.

Table 8A, 8B: was done in collaboration with Arnab Mukhopadhyay.

Table 9A, 9B: was done in collaboration with Arnab Mukhopadhyay.

Table 10: Strains were generated by me unless otherwise indicated.

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Abstract

The insulin/IGF-1 signaling (IIS) was initially identified in *C. elegans* to control a developmental phenotype called dauer. Subsequently, it was realized that lifespan was extended by mutations in this pathway and became an intense focus of study. The IIS pathway regulates growth, metabolism and longevity across phylogeny and plays important roles in human disease such as cancer and diabetes. Given the large number of cellular processes that this pathway controls, understanding the regulatory mechanisms that modulate insulin/IGF-1 signaling is of paramount importance.

IIS signaling is a very well-studied kinase cascade but few phosphatases in the pathway are known. Identification of these phosphatases, especially those that counteract the activity of the kinases, would provide a better insight into the regulation of this critical pathway. Study of serine/threonine phosphatases is hampered by the lack of appropriate reagents.

In Chapter II, we discuss the design and results of an RNAi screen of serine/threonine phosphatases performed in *C. elegans* using dauer formation as a phenotypic output. We identified several strong regulators of dauer formation and in Chapter III, proceed to characterize one of the top candidates of our screen, *pptr-1*. We show that *pptr-1* regulates the IIS and thereby affects lifespan, development and metabolism in *C .elegans*.

pptr-1 gene encodes a protein with high homology to the mammalian B56 family of PP2A regulatory subunits. PP2A is a ubiquitously expressed phosphatase that is involved in multiple cellular processes whose specificity determined by its association with distinct regulatory subunits.

Our studies using *C. elegans* provides mechanistic insight into how the PP2A regulatory subunit PPTR-1 specifically modulates AKT-1 activity by regulating its phosphorylation status in the context of a whole organism. Furthermore, we show that this mechanism of regulation is conserved in mammals.

CHAPTER I

A general introduction to aging and its study

Introduction:

Aging has been one of the most accepted but least understood processes in human life. Aging can be defined as an inevitable decline in physiological functions over time affecting almost all organisms. Some of the universal features of aging include decrease in metabolic activity, decrease in the ability to reproduce and physiological deterioration of tissues(Browner, Kahn et al. 2004) (Figure 1). Every tissue, organ and organ-system becomes involved in this interminable process until the whole organism fails, resulting finally in the death of the individual(Leveille 2004; Volpi, Nazemi et al. 2004; Wolkow 2006). The physiological decline associated with aging results in increased incidence of age related diseases such as hypertension, diabetes, degenerative diseases like arthritis, stroke and dementia.

Surprisingly some of the features of aging are remarkably similar in diverse species. For instance, mitochondrial activity and metabolism has been shown to reduce as a function of age in humans and other model organisms like flies and worms(Ames 2004; Balaban, Nemoto et al. 2005). Aging *C. elegans* show muscle wasting, slow mobility and similar histological features of sarcopenia (muscle wasting) that are commonly seen in older individuals(Fisher 2004; Wolkow 2006).

Aging is one of the foremost causes of disease and decline in our society. The risk of diabetes, cancer, cardio-vascular and other major diseases all increase with age. The top five morbidities that increase as a result of aging are cardiovascular disorders, dementia and nervous system disorders, musculoskeletal disorders, infectious diseases and neoplasia (Committee on a National Research Agenda on

Aging 1991). Improvements in health care over the past few decades have resulted in an expansion of the geriatric population and a concomitant increase in health-care needs. Over 50% of the health care budget is projected to be used for age-related disorders over the next decade and with a projected $3.4 trillion health budget by 2014 (S. Heffler; Rice and Fineman 2004; Gandjour 2009), the immensity of the problem is obvious. Therefore, an insight into the process of aging that helps to reduce/mitigate age related decline is bound to eventually translate into almost every field of modern medicine and science.

Postulated mechanisms of Aging:

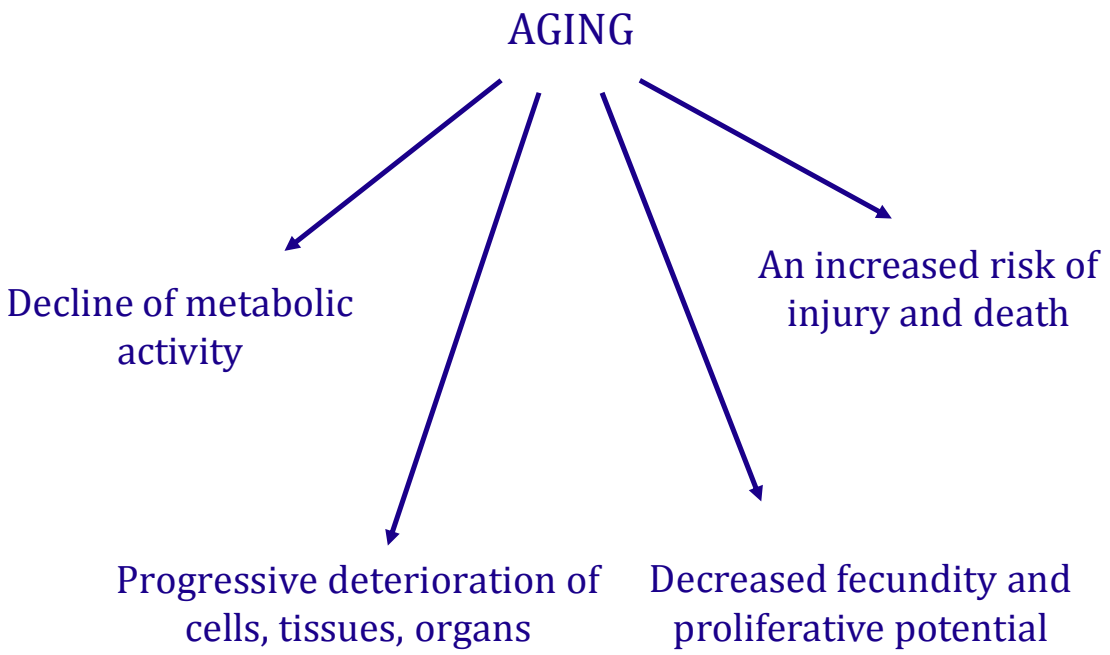
More than a dozen different theories have been put forward as probable causes of aging (causal) and all of these can be broadly classified into 2 main groups(Ricklefs 1998; Moorad and Promislow 2008; Ljubuncic and Reznick 2009). The basic tenet of the first group (Genetic theories of aging) is that aging is a genetically programmed parameter much like an extension of development and puberty. The second group (Wear and tear theories of aging) postulates that aging is a result of normal wear and tear of living, a result of both internal and environmental insults, as a byproduct of life-processes.

Causal Theories of aging:

Wear and Tear theories:

Contrary to the genetic theories of aging, the proponents of the Damage (Wear and tear) group of theories contend that aging is a result of irreversible accumulation of damage over time that finally results in death. The most commonly used analogy is to that of an old car. After multiple repairs there reaches a point in the life of a car that multiple systems start to fail and it is no longer feasible to repair the vehicle anymore. Mathematicians have systematically studied and modeled the failing of mechanical devices, formally known as ‘Reliability Theory’. Surprisingly enough, the Weibull distribution that predicts

What is aging?



Adapted from Braechman *et al.* (2001) *Mech. Ageing and Dev.*

Figure 1

mechanical failure rates can also accurately model mortality data of a population. The wear and tear theories implicate various sources of damage as the main reason for the aging process.

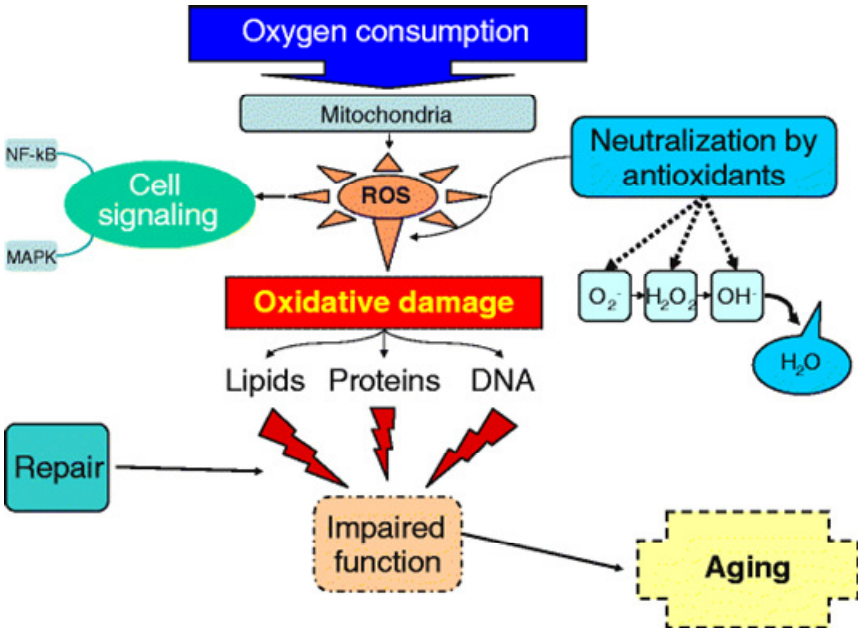
The Oxidative damage or free radical theory (Buffenstein, Edrey et al. 2008) was originally proposed by Herman in 1950 . Free radicals are highly reactive molecules generated as a byproduct of redox reactions in the cell. The major cellular source of free radicals is derived from oxygen and termed Reactive Oxygen Species (ROS). ROS can react with cellular macromolecules such as DNA, RNA, protein and lipid resulting in damage and interference with their normal function (Figure 2). Free radical damage to DNA can result in cross-linking of DNA strands, base substitutions and deletions resulting in mutation and genomic instability. Amino acids in structural proteins and enzymes can undergo covalent modifications (for example, protein carbonylation) that interfere with normal function of the protein. Lipofuscin (intracellular aging pigment) is known to accumulate in lysosomes as a function of age and has been shown to be an indigestible byproduct of unsaturated fatty acid oxidation. In order to prevent such damage, cellular mechanisms exist that counteract and neutralize ROS. Enzymes such as catalase, superoxide dismutase and proteins like glutathione aid in scavenging free radicals before they damage cellular components.

The oxidative damage theory has also resulted in several derivative theories of aging. Although ROS can be produced in diverse ways including ionizing radiation, peroxisomal fatty acid oxidation, and other cellular redox reactions, the major source of ROS is from the mitochondria as a byproduct of ATP production. This has resulted in the mitochondrial theory of aging and the related rate of living theories. Since the mitochondria are the seat of oxidative phosphorylation and consequently ROS production they are also the most affected by oxidative damage. Indeed, mitochondrial DNA damage has been documented

to increase as a function of age. However, there has been no conclusive proof of mitochondrial damage in the causality of aging.

Cellular damage in particular has been proposed to be an important determinant in the aging process. In order to maintain cellular homeostasis, damage response and repair pathways exist in the cell. DNA damage can be corrected by DNA repair enzymes and damaged proteins can be targeted to undergo recycling by protein degradation pathways. The mutation accumulation (DNA Damage) theory in particular posits that mutations in DNA accumulate as a function of time, until finally the damage exceeds the repair limits of the cell and results in a catastrophic failure and death. However, studies have shown that the mutation rates in old animals are not very different from those of young animals. Moreover, cells in old animals do not show a high enough level of mutations in order to justify the catastrophic mutation theory. As of now, there is no firm proof that DNA damage and mutation accumulation alone are responsible for the general aging seen in most organisms.

Despite this view however, the role of DNA damage in specific cellular niches cannot be completely dismissed. Most tissues in mammals and other multicellular organisms are in a state of constant renewal from stem cells. Any damage in this small but important compartment of cells can have an impact in the overall health and state of the organism(Bellantuono and Keith 2007; Ljubuncic and Reznick 2009). This could especially be true in some of the inherited accelerated aging syndromes like Werner's and Hutchinson-Gilford progeria (Oshima 2000; Bridger and Kill 2004; Lans and Hoeijmakers 2006). The affected individuals develop several phenotypes of accelerated aging including the appearance of cardiovascular diseases and failure to thrive resulting in a severely compromised lifespan. One of the reasons for the accelerated aging phenotype in these accelerated aging syndromes is thought to be a result of selective depletion of stem-cell population as a result of increased DNA damage and mutational load(Brown 2003; Kaneko and Kondo 2004; Beausejour 2007; Bellantuono and Keith 2007).



Oxidative damage as a cause of aging

(Buffenstein, Edrey et al. 2008)

Figure 2

The telomerase theory of aging, when it was proposed, seemed to be an elegant solution to the long standing quest to understand aging. Eukaryotic genome is organized into highly packaged linear chromosomes. DNA polymerases are unable to completely replicate the 3' end of linear DNA and as a result with successive replications the ends of linear DNA will tend to be lost. The ends of linear chromosomes are therefore protected by repeats called telomeres. Telomeres are added to the ends by the enzyme telomerase. Interestingly, telomerase activity is only present in stem cells and transformed cancer cells but absent in somatic cells. Since somatic cells do not express telomeres, with each successive division they lose a small part of the telomere until they reach a point (Hayflick limit) when the telomeres are completely lost and DNA damage begins to occur and cells undergo senescence and stop dividing. The telomerase theory suggests that aging is because of successive shortening of telomeres with repeated cell divisions thereby eventually resulting in senescence. However, several studies have challenged the importance of telomerase in normal aging. Firstly, there was no significant difference in the replicative lifespan of fibroblasts derived from old or young individuals and secondly mouse studies have shown that telomerase knockouts did not show any difference in aging or lifespan compared to wild-type mice. These and other studies have therefore concluded that currently there is no clear-cut evidence that the length of telomeres has any correlation to normal aging processes and phenotypes.

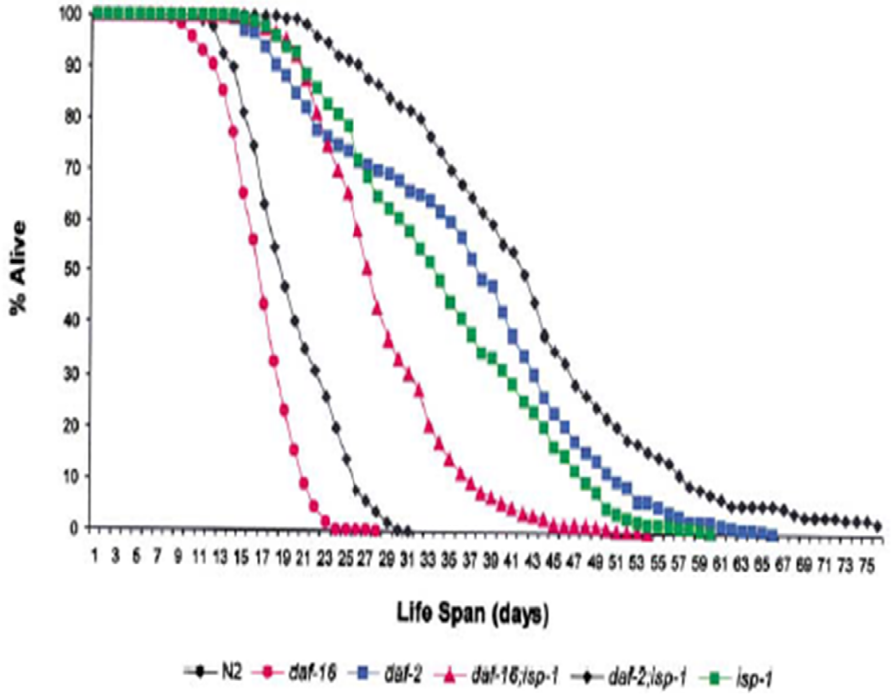
The rate of living theory suggests that every organism has a limited maximal aerobic potential which is determined by the mitochondrial capacity for oxidative phosphorylation. Studies in flies and worms have shown that the lifespan of these organisms are longer at a lower temperature, where in the oxygen consumption and rate of metabolism are reduced. Similarly, an inverse correlation has been observed between metabolic rate and lifespan in different species. Smaller mammals seem to have higher metabolic rates and shorter lifespans compared to larger mammals with longer lifespans. However, there are several exceptions to this general rule, for example, birds and bats have a much higher metabolism and

longer lifespan than similar sized rodents suggesting that the observed correlation between metabolic rate and longevity might be a result of independent evolutionary pressures on lifespan and metabolic rates.

Genetic theories of aging:

The phenotypic similarity of aging individuals within a species coupled with the species specific restriction of maximum lifespan lends credence to the idea that aging is probably an extension of development. The genetic/programmed theories were a subject of great controversy in the past but recent data from model organisms has renewed interest in them. In C. elegans, for example, mutations have been identified that extend the lifespan by almost 5 times the wild-type lifespan (Figure 3). Interestingly, most of the well studied genes that affect lifespan also affect the developmental cycle of *C. elegans.*

C elegans undergoes 4 larval molts L1 through L4 before becoming an adult in the presence of conducive environmental conditions. However, under adverse conditions like high temperature, overcrowding and starvation they enter an alternative larval stage (L2d) called dauer. Dauer formation is controlled by several distinct pathways of which the major pathway is insulin/IGF-1 signaling cascade. Mutations in certain components of insulin/IGF-1, a neuroendocrine signaling pathway, can result in increased mean and maximal lifespan. Data in worms suggest that signals from diverse tissues like sensory neurons, the gonad and the germline converge on the insulin/IGF-1 pathway to eventually modify the lifespan of the whole organism. Surprisingly, mutations that interfere with IGF1 signaling in mice also enhance longevity. For example, Ames dwarf mice have a mutation in *Prop-1* that results in a defect in anterior pituitary differentiation. These mice have lower levels of circulating GH and IGF1 but live almost 50% longer than wild-type mice(Barbieri, Bonafe et al. 2003; Holzenberger, Dupont et al. 2003). Exciting findings such as these have resulted in the resurgence of interest in role of genetics in aging. Current data



Maximum lifespan has been increased by upto 5 times in *C. elegans* by muations

Figure 3

Feng, J., Bussiere, F. & Hekimi, S. Developmental Cell (2001)

from worms, flies, mice and other animals indicate that aging can be modulated in these organisms by genetic manipulations. However, no gerontogene has been identified that can conclusively change the rate of aging and therefore no firm proof exists that aging is a strictly genetically regulated phenomenon.

None of the afore-mentioned theories, can completely explain the current data we have from model organisms, epidemiological and evolutionary studies. Aging seems to be a multifaceted problem with genetic, environmental and stochastic components and no single theory of aging can completely explain all its features.

Studying aging:

Biomarkers:

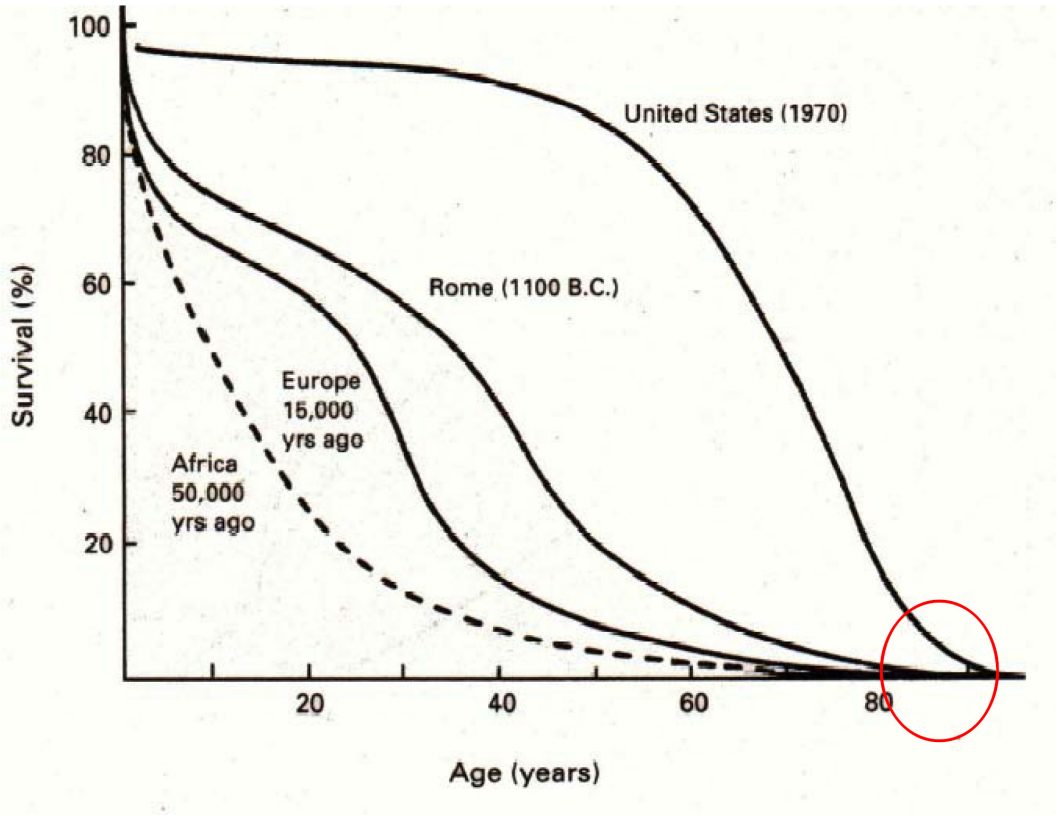
An important requirement in aging studies is to predict the rate of aging. Aging is inherently a stochastic process, but can be significantly modulated by genetic and non-genetic (dietary restriction) interventions(Austad 2001). Different individuals of the same chronological age in a population show significantly different physiological states necessitating a way to measure the physiological age of the individual. Also any intervention that increases lifespan significantly, probably, does so by delaying or slowing aging. A biomarker of aging must therefore be a direct indicator of physiological age. It must show a slower change or accumulation in treatments enhancing longevity and vice versa(McClearn 1997; Ingram, Nakamura et al. 2001). Identifying biomarkers of aging has an enormous potential for accelerating aging research since currently the only reliable indicator of aging is lifespan which is too tedious a parameter to follow and conduct elaborate experiments, more so in longer lived species. Although an almost decade long effort has identified several surrogate markers of aging (glycoxidation of collagen, cell replication, immunological markers) none of them are accurate enough to be used as a biomarker of aging in humans or in most other higher mammals {(Butler, Sprott et al. 2004; Warner 2004) }.

Lifespan/Mortality curves:

The most accepted measure of aging is lifespan(L. Piantanelli 1992; Johnson 2006). The lifespan of a single individual is simply measured as the length of time that the individual is alive. When such data is collected for multiple individuals of a particular species and plotted over a period of time, we get a remarkably reproducible parameter – the species specific lifespan. Generally the word 'Lifespan' in literature refers to the species specific lifespan unless qualified otherwise. In captive/laboratory/civilized conditions the slope of the lifespan curve is initially parallel to the x-axis where majority of the individuals are alive. This initial part of the curve can sometimes slope down more acutely in the presence of predation and disease (Figure 4). The second part of the curve is marked by an exponential drop in survival (and an increase in mortality) followed by a third slow (linear) drop-off when only a few long lived individuals are left until finally all of the individuals are dead (L. Piantanelli 1992). There are several features of the lifespan curve worth mentioning:

1) The overall shape of the lifespan curve of different species is similar (Figure 5) although the maximum lifespan of these species can differ by a factor of almost 10^3. This similarity in shape indicates a basic conservation of processes responsible for regulating longevity (or mortality) across phylogeny(Hughes and Reynolds 2005).

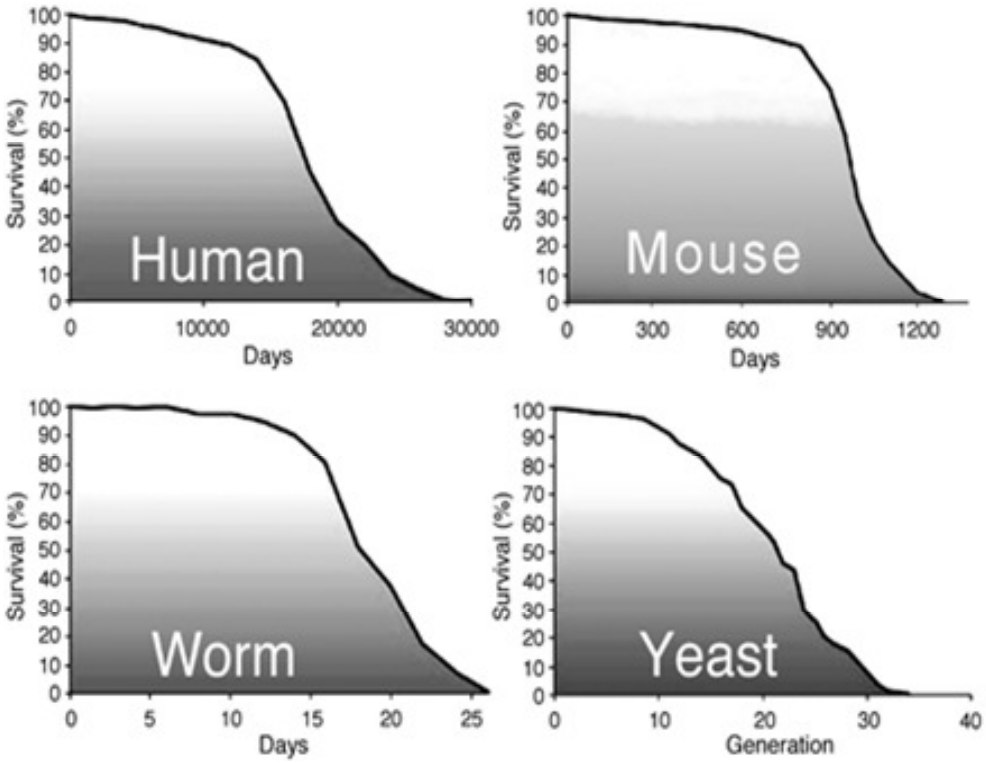
2) Maximum (Species specific) lifespan is constant for a given species. For example, over the past 50,000 years the maximum lifespan of humans has remained constant (Figure 4). But, the average lifespan (life expectancy) has improved significantly due to civilization and improvements in health-care as illustrated in Figure 4. The slope of the first part of the curve becomes more horizontal with decrease in age-independent causes of mortality. However, the second component of age-dependent mortality and as a result maximum lifespan has not changed significantly. This restriction of maximum lifespan despite



Maximum lifespan of humans has not changed over 50,000 yrs

Biology of Human Aging. Alexander P. Spence Prentice Hall, 1999

Figure 4



Similar survival curves of diverse species

Troen *et al.*, 2003 (from wormchip.stanford.edu)

Figure 5

increase in life expectancy indicates that processes responsible for regulating longevity is determined genetically. Not surprisingly therefore, in model organisms genetic mutations have been identified that can extend the maximum lifespan by as much as five-times (Figure 3)(Arantes-Oliveira, Berman et al. 2003).

3) Lifespan curve of a genetically isogenic population is indistinguishable from captive wild-type populations. As an example, lifespan curves of *C. elegans* (that can be propagated from a single hermaphrodite animal) and in-bred mouse lines still shows the same classical sigmoidal shape and age-dependent mortality as wild-type animals. This indicates that despite the presence of a genetic restriction on maximum longevity for a species, individual lifespan is determined by other stochastic/environmental factors (Wolkow 2006).

Model organisms for Aging studies:

One the most reliable and widely accepted parameter in the study of aging is lifespan. Lifespan has been shown to correlate well with aging process(Warner 2004). However, to measure lifespan it becomes necessary to follow a population till a significant end-point such as the mean (50% of individuals are dead) or maximum lifespan (all individuals of the population are dead) of that organism. Given the relatively long lifespan of humans it becomes necessary to use model organisms to study this process. Moreover, it is not possible to utilize systematic interventional studies for ethical and practical reasons in humans. Aging studies have therefore been done in model organisms to circumvent these problems. Studies have used unicellular organisms like yeast; invertebrate species like drosophila, *C. elegans*; and mammals such as mouse and monkeys(Kuningas, Mooijaart et al. 2008). The two key questions to be balanced with the use of model systems are:

1) How well does the underlying mechanism of aging that is being studied correlate (similar) with Humans?
2) How long (feasible) does it take for a study to determine if an intervention affects aging?

Feasibility is a direct correlation of several factors such as the time taken for the study, ease of maintaining the model organism and ease of the proposed intervention. Evolutionarily lower organisms are therefore more 'feasible' to study but evolutionarily closer organisms are more likely to have similar underlying mechanisms of aging compared to distant organisms. There are two potential solutions to this conundrum. First, is to utilize a surrogate of aging, such as biomarker, for studies in higher organisms, or second, use the more tenable models to systematically identify interventions/genes that regulate aging and then verify them in higher organisms. The latter approach has given many key insights into our current understanding of aging(Dufour and Larsson 2004).

Table 1 shows a comparison of different phenotypes associated with age between different model organisms. Several key features of aging such as mitochondrial dysfunction, muscle-loss and general decrease in fitness are relatively well conserved among different model organisms. Such processes are likely to be a result of 'universal' or conserved 'public mechanisms' of aging and studying them may yield important insights into human aging and disease(Hekimi 2006). However, it is important to identify and account for key differences that exist by virtue of evolutionary divergence. For example, cancers are a major cause of age associated mortality and morbidity in humans but the model organism *C. elegans* is a post mitotic and therefore immune to neoplasia.

C. elegans as a model organism for aging:

C. elegans has been used for over a decade to investigate the molecular mechanisms of aging. *C. elegans* has several inherent characteristics that make it an ideal model system. Powerful genetics (molecular and classical), small size, easy maintenance, transparency, well studied anatomical structure and complete sequence information with up-to-date annotation makes it an ideal system for investigating basic questions. However, apart from these general characteristics there are other compelling reasons for using it to study aging. A short lifespan of about 16 days at 25^{o}C makes lifespan experiments feasible. Lifespan is demographically similar to outbred, higher organisms (Figure 5). *C. elegans* shows age related changes like

lipofuschin accumulation, sarcopenia and above all random and highly idiosyncratic decline in tissue function that is very similar to that seen in mammals (Gershon and Gershon 2002; Herndon, Schmeissner et al. 2002; Fisher 2004).

It was predicted that metabolism and mitochondrial function were important determinants of metazoan lifespan. However, conclusive evidence came forth from *C. elegans* screens showing that reducing mitochondrial function (ETC – Electron Transport Chain) significantly extended lifespan (Sohal and Weindruch 1996; Feng, Bussiere et al. 2001; Lee, Lee et al. 2003). Similarly, much of our present understanding about the molecular mechanisms of aging comes from studying *C. elegans*. For example, the role of insulin-like signaling pathway in lifespan was initially identified in worms and later was confirmed in mammals (Kenyon, Chang et al. 1993; Brown-Borg, Borg et al. 1996; Henderson and Johnson 2001; Tu, Epstein et al. 2002; McCulloch and Gems 2003). In worms, knockout of *daf-2* (the insulin-like receptor) doubles lifespan while knocking out *daf-16* also, (a forkhead transcription factor) which is the final output of the pathway, abrogates the extension in lifespan (Gottlieb and Ruvkun 1994; Larsen, Albert et al. 1995; Ogg, Paradis et al. 1997). These among other studies have established *C. elegans* to be a viable system to study aging and still apply these findings to higher organisms.

Aging phenotypes in Model organisms.

Phenotype	*H. sapiens*	*M. musculus*	*D. melanogaster*	*C. elegans*
Decreased cardiac function	Yes	Yes	Yes	NA
Apoptosis, senescence (somatic cells)	Yes	Yes	Yes	?
Cancer, hyperplasia	Yes	Yes	No	No
Genome instability	Yes	Yes	Yes	Yes
Macromolecular aggregates	Yes	Yes	Yes	Yes
Reduced memory and learning	Yes	Yes	Yes	NA
Decline in GH, DHEA, testosterone, IGF	Yes	Yes	?	?
Increase in gonadotropins, insulin	Yes	Yes	?	?
Decreased thyroid function	Yes	Yes	NA	NA
Decrease in innate immunity	Yes	Yes	Yes	Yes
Increase in inflammation	Yes	Yes	No	No
Skin/cuticle morphology changes	Yes	Yes	?	Yes
Decreased mitochondrial function	Yes	Yes	Yes	Yes
Sarcopenia	Yes	Yes	Yes	Yes
Osteoporosis	Yes	Yes	NA	NA
Abnormal sleep/rest patterns	Yes	Yes	Yes	?
Decrease in vision	Yes	Yes	?	NA
Demyelination	Yes	Yes	?	No
Decreased fitness	Yes	Yes	Yes	Yes
Arteriosclerosis	Yes	No	NA	NA
Changes in fat	Yes	Yes	?	?

Table 1

(Vijg and Campisi 2008)

Insulin/IGF-1 signaling pathway in *C. elegans*:

The insulin/IGF-1-like signaling (IIS) pathway is an evolutionarily highly conserved neuro-endocrine pathway that regulates multiple biological processes including metabolism, development, stress resistance and lifespan (Finch and Ruvkun 2001; Barbieri, Bonafe et al. 2003; Kenyon 2005; Wolff and Dillin 2006; Antebi 2007). Almost every component of the IIS pathway found in *C. elegans* has a homologue in mammals with a similar conserved function (Figure 6). However, keeping with the exponential increase in complexity up the evolutionary ladder this pathway has greatly expanded.

In mammals there are two distinct pathways – the insulin pathway dedicated to metabolic/energy regulation and the IGF-1/2 pathway that regulates growth and survival. The upstream components in humans include the 3 ligands (insulin, IGF-1 and IGF-2) and 3 receptors (Insulin receptor, type-1 and type-2) to which they bind. The insulin receptor is a heterotetramer consisting of two α and β subunits coupled by disulphide linkages. Ligand binding to the extra-cellular α subunits results in a conformational change that propagates to the transmembrane β -subunits and activates its intrinsic tyrosine kinase activity. Activation of tyrosine kinase activity then results in autophosphorylation of key residues in the intracellular domain and downstream targets (including the insulin receptor substrates –IRS proteins) that mediate downstream signaling from the receptor. Phosphorylation of IRS proteins then allow them to associate to SH2 domain containing proteins such as the PI3 Kinase regulatory subunit (activating PI3 Kinase pathway) and GRB2, SHP2 (activating ras/MAPK pathway) thus allowing signals to propagate to multiple downstream pathways.

The PI3-Kinase pathway has been recognized to be an important component of the insulin/IGF-1 signaling pathway and known to mediate glucose transport, glycogen storage, growth stimulation and survival. Activation of the PI3 Kinase in turn recruits PI3-dependent kinase (PDK1), a serine/threonine

The Insulin/IGF-1 pathway is conserved

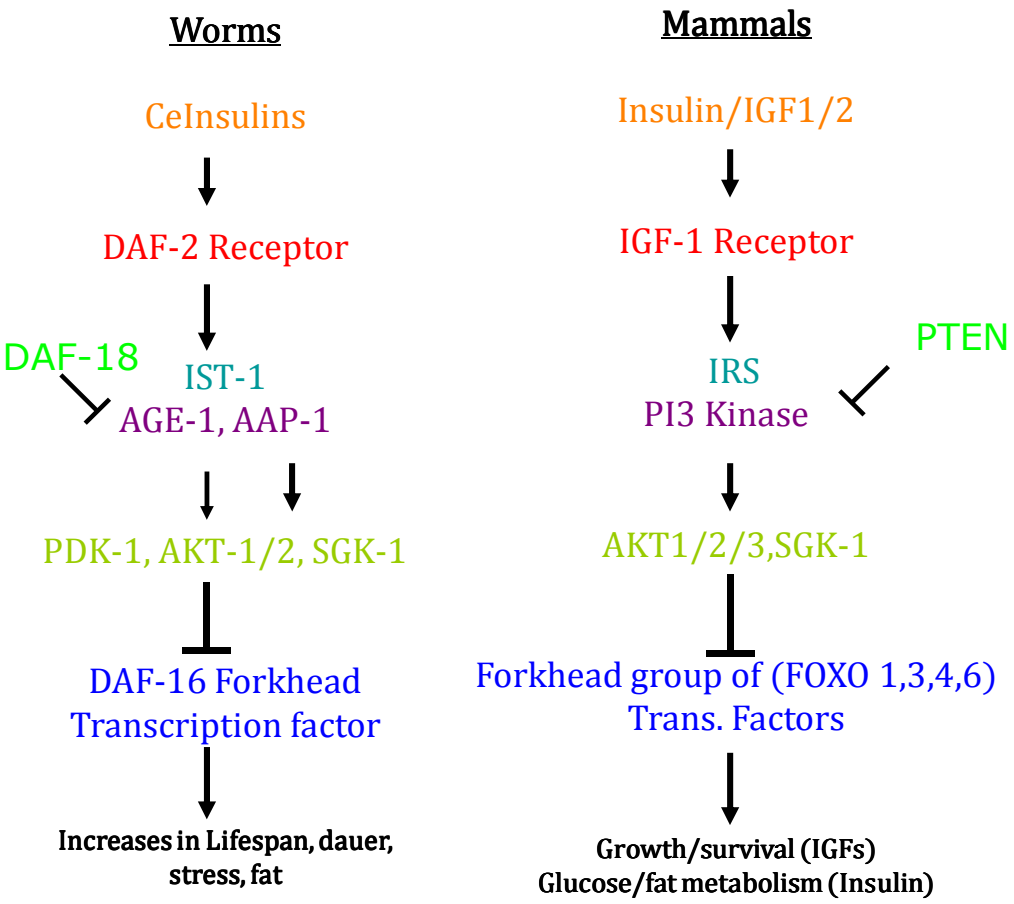


Figure 6

kinase, which then in turn phosphorylates and activates other downstream serine/threonine kinases of the AGC-family (AKT1/2/3,SGK1, p70rsk and PKC), these in turn phosphorylate their substrates to regulate diverse responses such as growth, differentiation, glucose transport, angiogenesis and stress response (Barbieri, Bonafe et al. 2003; Giri, Mutalik et al. 2004; Leibiger and Berggren 2008; Salih and Brunet 2008).

In *C. elegans*, the insulin-like receptor DAF-2 (Kimura, Tissenbaum et al. 1997) signals through a PI 3-kinase (AGE-1/AAP-1) (Morris, Tissenbaum et al. 1996; Wolkow, Munoz et al. 2002) signaling cascade that activates the downstream serine/threonine kinases PDK-1, AKT-1, AKT-2 and SGK-1 (Paradis and Ruvkun 1998; Paradis, Ailion et al. 1999; Hertweck, Gobel et al. 2004). These kinases in turn function to negatively regulate the forkhead transcription factor (FOXO), DAF-16 (Lin, Dorman et al. 1997; Ogg, Paradis et al. 1997). Reduction-of-function mutations in serine/threonine kinases upstream of DAF-16 leads to changes in lifespan, development, metabolism and/or stress resistance (Kenyon 2005; Wolff and Dillin 2006; Antebi 2007). Genetic studies in *C. elegans* showed that loss-of-function mutations in *daf-16* completely suppresses these phenotypes (Kenyon 2005; Mukhopadhyay, Oh et al. 2006; Wolff and Dillin 2006; Antebi 2007) implicating DAF-16 to be a major downstream target of the IIS pathway in worms.

Serine/threonine phosphorylation of distinct sites in DAF-16 by AKT-1, AKT-2 and SGK-1 results in its nuclear exclusion and sequestration by 14-3-3 proteins in the cytosol (Lin, Hsin et al. 2001; Hertweck, Gobel et al. 2004). In contrast, under low signaling conditions, DAF-16 is relieved of the inhibitory signals to enter the nucleus and regulate its target genes (Henderson and Johnson 2001; Lee, Hench et al. 2001; Lin, Hsin et al. 2001; Hertweck, Gobel et al. 2004; Oh, Mukhopadhyay et al. 2006). negative regulation of FOXO/DAF-16 by upstream kinases is conserved across species. Studies in mammals have shown that the mammalian AKT and SGK kinases can phosphorylate and negatively regulate FOXO (Brunet, Bonni et al. 1999; Brunet, Datta et al. 2001; Brunet, Park et al. 2001; Calnan and Brunet 2008).

In mammals FOXO has been extensively studied and known to play key roles in cell-cycle arrest, stress response, differentiation and metabolism. Some of well studied down-stream targets of FOXO family

members include mediators of stress resistance (MnSOD, Gadd45α) (Salih and Brunet 2008), angiogenesis (Ang2,eNOS), Apoptosis (Bim, fas), metabolism (Ppargc1α, G6pc) and cell cycle arrest($p27^{Kip}$,$p21^{Cip}$). These endogenous functions of FOXO protein make it a very important target in neoplasia. Mutations in PTEN in breast cancer cells act by sequestering FOXO3 cytosol and preventing its activity. Expression of active FOXO3 results in inhibition of tumor growth in-vivo(Carter and Brunet 2007). Such studies have established the importance of FOXO proteins as important downstream mediators of insulin/IGF-1 signaling in mammals. It is also thought that FOXO activity might also be important in mammalian longevity given the similarity in the classes of downstream targets(Barbieri, Bonafe et al. 2003; Amador-Noguez, Yagi et al. 2004). However, it is not currently known which, if any, FOXO isoforms are required for the long-lived phenotype of IGF-1 signaling defective mice models (Barbieri, Bonafe et al. 2003; Holzenberger, Dupont et al. 2003).

In worms, as described above, DAF-16 is known to be absolutely required for multiple phenotypes of insulin pathway mutants including lifespan extension. Multiple studies have been done to identify DAF-16 targets, particularly those responsible for the lifespan extension in IIS mutants. More than a few hundred genes were identified with some of the major classes being antioxidant genes(*sod-3, ctl-1*), stress response (*hsp-16, hsp-12.6*), pathogen resistance (*spp-1*), metabolic (*gei-7, pnk-1*) and anti-toxicity (*mtl-1)*. From these results it appears that DAF-16 regulates a large group of genes in concert to mediate its longevity, stress resistance and other phenotypes(Hsu, Murphy et al. 2003; Lee, Kennedy et al. 2003; Murphy, McCarroll et al. 2003; Jensen, Gallo et al. 2006; Oh, Mukhopadhyay et al. 2006). The potential implication of these studies is that to modulate organismal aging successfully interventions must target upstream regulators of IIS/DAF-16 to activate the endogenous longevity assurance programs. In this light, identifying regulatory components of IIS/DAF-16 are mission critical for not only unraveling the complexity of aging but also to allow intervention in this process. In addition, such regulators will also be important

drug targets for a multitude of human diseases such as diabetes, dementia and cancer wherein dysregulated insulin/IGF-1 signaling is implicated.

CHAPTER II

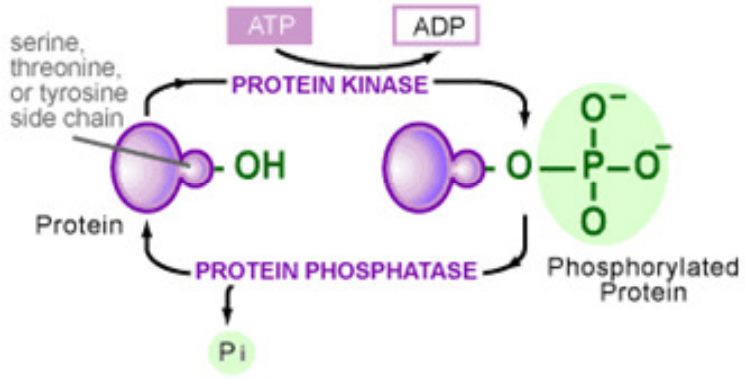
RNAi Screen for serine/threonine phosphatases regulating the C. elegans insulin/IGF-1 signaling cascade

Introduction :

Regulation of Protein function:

Living organisms need to regulate their internal processes in a concerted and meaningful fashion in order to survive in an ever changing world. In order to do so, proteins can be regulated at the level of transcription, translation and post-translation. In eukaryotes, one of the most common means of regulating protein function is by post-translational modifications (PTM). There are several known PTMs such as acetylation, alkylation, amidation, sulfation, glycosylation (addition of glucose/carbohydrate moiety), prenylation (addition of farnesyl or geranylgeranyl isoprenoids derived from cholesterol), glutamylation, phosphorylation, ubiqination (ubiquitin peptide) and sumolyation. Some PTMs like glycosylation (cell surface and secreted proteins) and glutamylation (clotting factors) are essential to the normal activity of the protein. Others can result in enhancing or attenuating activity (phosphorylations), membrane localization (prenylation), and even target proteins to degradation (ubiquination). PTMs are not only used for regulating the activity of proteins but also for transducing extracellular signals into the cell quickly and reliably to target specific internal processes. Recently it has been appreciated that the same residue can be subject to different PTMs. The classical example is the 'Yin-Yang' model of O-Glc-Nac (glycosylation) modification blocking a phosphorylation at the same phospo-acceptor residue. This in-effect causes one modification to block the effect of the other ('Yin-Yang' model) and in some cases can result in complex effects including completely different cellular responses(Slawson, Housley et al. 2006).

A



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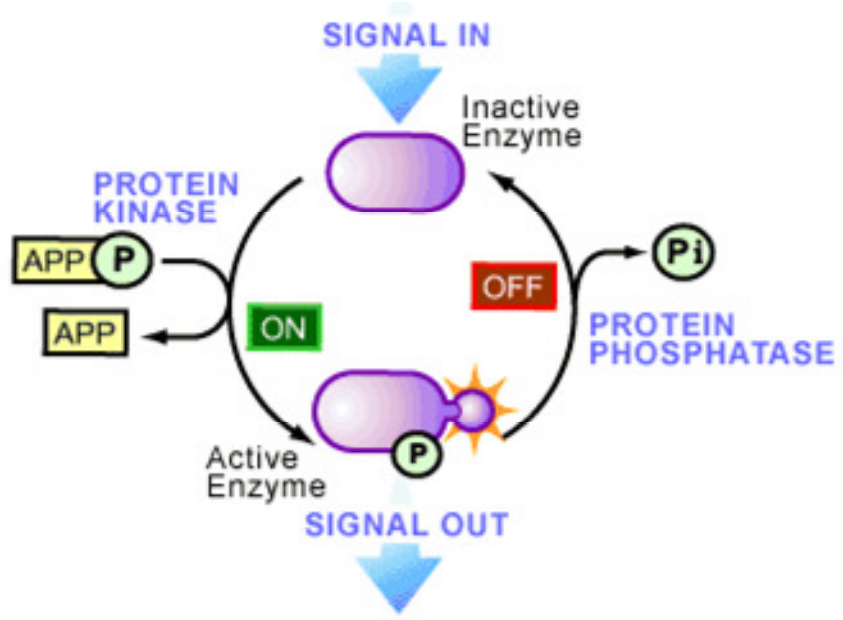

A) Schematic of classical kinase / phosphatase reaction

B) Schematic of a signaling module mediated by reversible phosporylation

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Figure 7

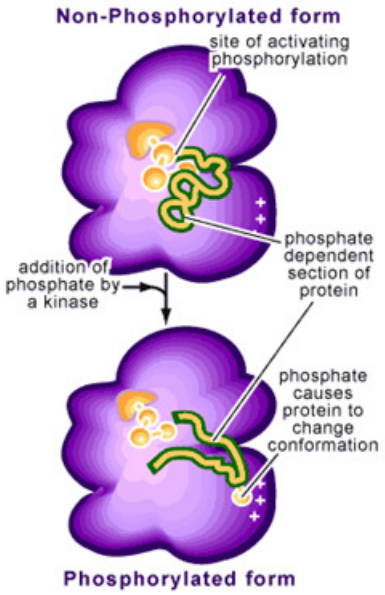
Protein Phosphorylation:

One of the most well known and well studied PTM is reversible protein phosphorylation. Protein phosphorylation is catalyzed by enzymes (kinases) that hydrolyze ATP and transfer the γ-Phosphate from ATP to specific residues in the protein substrate. The reverse hydrolysis of the phosphate moiety from the protein is catalyzed by hydrolases (phosphatases) that return the protein to its original state (Figure 7A). There are several advantages to using phosphorylation as a key PTM for the cell. Covalent addition of a phosphate to amino acid side chains is rapid (few milliseconds), easily reversible and as a corollary, very low cost to the cell. To illustrate, to synthesize a 100 aa protein the energy cost for the cell is around 400 high-energy phosphates, however, the same protein can reversibly undergo 400 phosphorylation-dephosphorylation reactions without any loss of activity. The popularity of phosphorylation is attested to by the fact that almost 3-5% of most eukaryotic genomes consist of kinases and phosphatases and nearly 30% of cellular proteins are phospho-proteins.

Although phosphorylation is a facile and simple modification, it can have profound effects on the function of the target protein. Addition of a phosphate group confers a net negative charge to the amino acid side chain to which it is added resulting in a variety of possible changes. For example, phosphorylation can trigger a change in the conformation of target proteins, modify activity of enzymes, regulate interacting partners, target intracellular localization, change the stability of the protein substrate and finally even transduce signals (examples: Figure 7B,Figure 8A).

Phosphorylation is employed extensively in signaling cascades which transfer an extracellular signal into the cell to modify functions/processes within. In signaling cascades many of the substrates that are phosphorylated in response to external signals are themselves kinases which are in-turn capable of phosphorylating other protein kinases/substrates. This cascading arrangement of kinases permits signal amplification and transmission through the cell to the final intended target(s) (Figure 8B).

A



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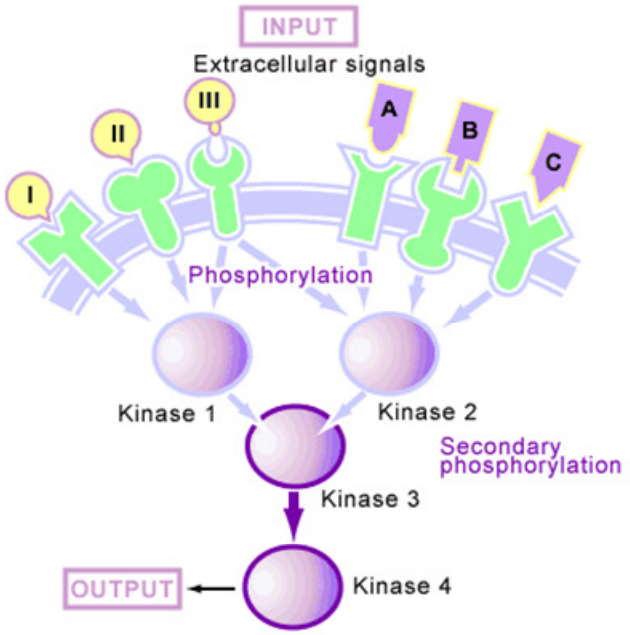

A) Schematic of phosphorylation activating an enzyme.

B) Schematic of successive kinases resulting in a signaling cascade.

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Figure 8

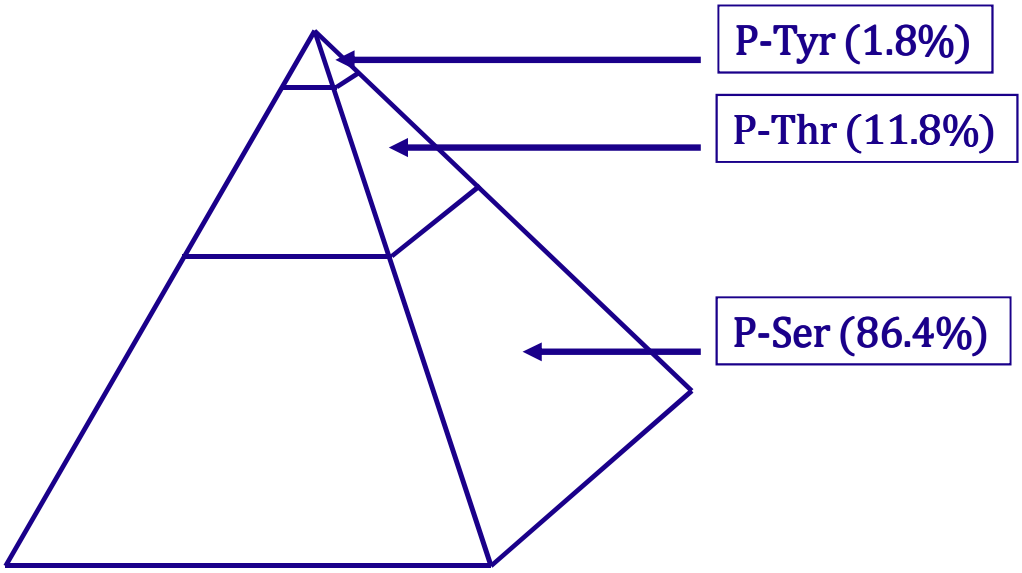
Generally, signaling cascades remain active as long as the phosphorylation events persist. For a meaningful regulation of cellular functions, the transmission of signals into the cell needs to shut off as soon as it is no longer needed. Phosphatases therefore become intrinsically important to counteract and terminate signals by dephosphorylation of the down-stream kinases (and their substrates) to return them to their normal state. Indeed mutations resulting in hyper-activated signaling of growth-factor pathways are implicated in a majority of cancers.

Proteins can be phosphorylated on nine amino acids: serine, threonine, tyrosine, cysteine, lysine, aspartate, glutamate and histidine. However the predominant phosphorylation events mediated by eukaryotic kinases occur at serine, threonine and tyrosine residues in that order (Figure 9). Based on the phospho-acceptor amino-acid in the substrate, kinases can be broadly classified into 3 groups. The two major groups are tyrosine and serine/threonine kinases and smaller group of dual-specificity kinases (for example, MAPKK) that can phosphorylate all three residues. Phylogenetically, all kinases share the catalytic domain (eukaryotic protein kinase - ePK motif) indicating that most kinases evolved from a common ancestor(Manning, Whyte et al. 2002). In humans there are approximately 518 kinases accounting for 1.7 % of the genome. Of these, 90 are tyrosine kinases (PTKs) and majority of the rest are serine/threonine kinases.

A short overview of protein phosphatases:

Phylogenetically, phosphatase evolution appears to have been more divergent and can be structurally and mechanistically classified into three major families (Tonks 2006; Moorhead, Trinkle-Mulcahy et al. 2007) (Table 2). The three classes are the PTP (phospho tyrosine phosphatase), PPP/PPM (both mostly phospho serine/threonine phosphatases) and finally the recently classified aspartate based catalysis group (FCP/SCP/HAD) group of enzymes.

Distribution of the Phosphate Group in Phosphoproteins



Modified from : EUROPHOSPHATASES 2003, Barcelona, Spain - Viktor Dombrádi

Figure 9

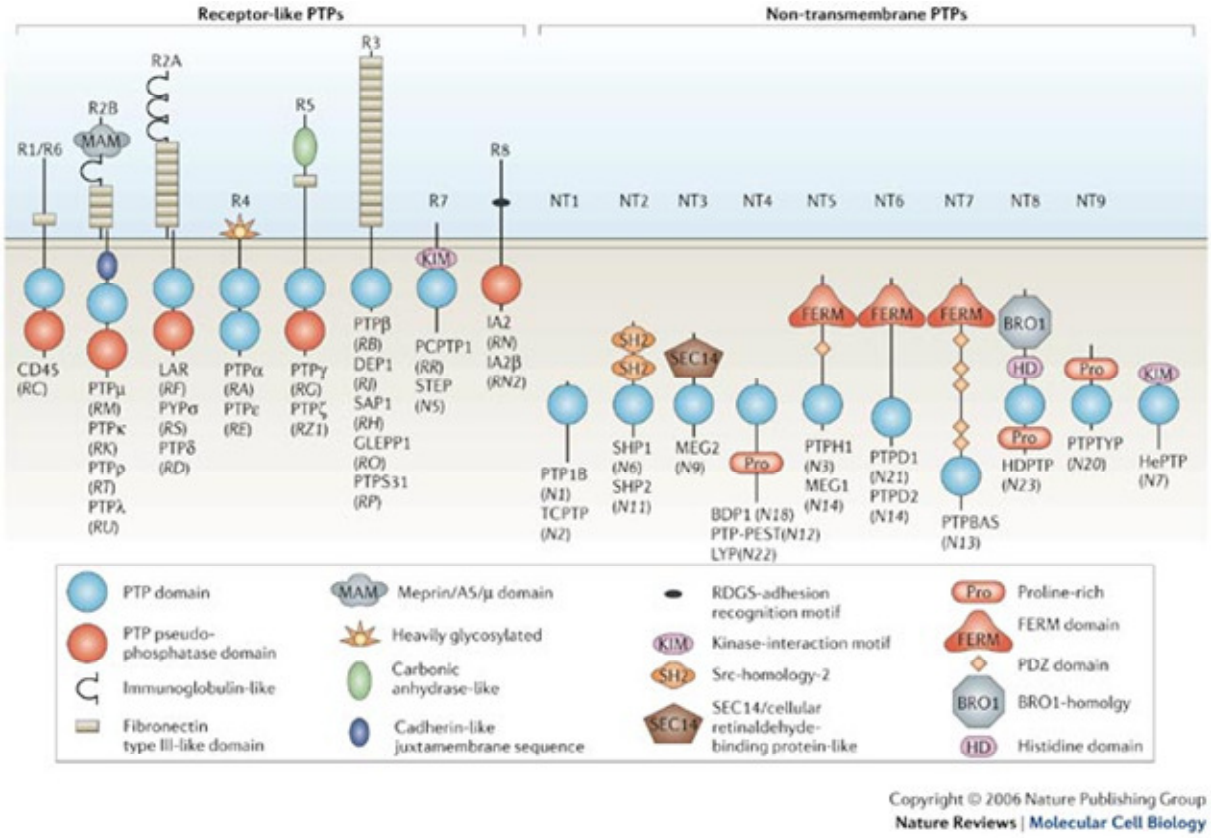
Phospho tyrosine Phosphatase family:

The PTP members are a diverse group of about 100 members in humans. The similar number of tyrosine kinases and PTPs in the human genome indicates that the complexity of these opposing groups of proteins is similar. All PTPs share the similar catalytic motif (CX5R) in their catalytic domains but have diverse functional domains and consequently different substrate specificities and affinities. Indeed, some family members of PTPs are now known to dephosphorylate carbohydrates, mRNA and phosphoinositides(Moorhead, De Wever et al. 2009). The diversity in domain composition among the PTPs indicate that they have evolutionarily incorporated catalytic and regulatory modules (mediating substrate specificity) into a single protein at the genomic level.

There are three main classes of PTPs (Figure 10). The first Class is further subdivided into the two main groups - the classical group of pTyr specific phosphatases and the Dual specificity phosphatases (DSPs). While the classical group of PTPs usually only dephosphorylate pTyr, the DSP group of enzymes are more pleiotropic and structurally diverse. DSPs have been shown to dephosphorylate serine, threonine, tyrosine, lipids and even complex carbohydrates(Tonks 2006; Moorhead, De Wever et al. 2009). Some important examples include the tumor suppressor PTEN (Phospho-inositol tri-phosphate phosphatase), MKPs (MAPK phosphatases – serine/threonine and tyrosine residues) and KAP (CDK associated phosphatase). The class II and Class III enzymes are represented by CDC25 and LMPTP respectively (Low mol. wt. phospho tyrosine phosphatase) and are thought to have evolved from primitive bacterial enzymes.(Alonso 2004; Andersen 2004)

The Classical tyrosine phosphatase members can functionally be further classified into two classes: the transmembrane receptor like proteins (R) and the non-transmembrane/cytosolic PTPs (NR). The R-PTPs regulate signaling upon ligand binding by dephosphorylation of protein pTyr in contrast to

Classification of PTPs and complexity of their domain architecture



Tonks *Nature Reviews Molecular Cell Biology* **7**, 833–846 (November 2006) | doi:10.1038/nrm2039

Figure 10

the well known receptor tyrosine kinases. The R-PTPs have been implicated in cell-cell contact, adhesion mediated signaling (CAMs) and also in matrix-cellular interactions. The NR-PTPs are cytosolic and contain the catalytic domain and associated specific regulatory domains that mediate substrate specific dephosphorylation. For example, SH2 domain-containing protein tyrosine phosphatase-2 (SHP2) is targeted to pTyr motifs by the SH2 domain and is involved in regulating ras/MAPK pathways upon growth factor signaling. (Neel, Gu et al. 2003; Salmond and Alexander 2006)

PPP/PPM families of Serine/threonine phosphatases:

In contrast to PTPs, the number of Serine/threonine phosphatases (PSTP) in the human genome is vastly smaller than the corresponding number of S/T Kinases. In humans, for example, there are more than 400 S/T kinases but only about 40 PSTPs(Barford D 1998; Cohen 2004; Moorhead, De Wever et al. 2009). Considered with the fact that more than 98% of the phospho proteins are modified at serine/threonine residues the question arises – how do such a small set of serine/threonine phosphatases regulate the great variety of substrates in an orderly and regulated fashion? The answer to this lies in the fact that most serine-threonine phosphatases are designed to function in a modular fashion. The phosphatase catalytic subunits associate with other proteins in the cell (regulatory proteins) which can then specify the appropriate substrate for the phosphatase. Indeed, numerous studies have shown that in-vivo, these phosphatases show exquisite specificity to substrates but in-vitro, and in the absence of the regulatory subunits, display promiscuous dephosphorylation towards substrates(Barford D 1998; Cohen 2004; Moorhead, De Wever et al. 2009).

PSTPs consist of the two distinct families: the PPP and PPM family of enzymes, based on sequence similarity. Initially, before sequence information was known, the key difference upon which they were grouped was based on the requirement of exogenous divalent metal ions (especially Mg^{2+} or Mn^{2+}) for the activity of PPM members and absence of such a requirement among PPP members. It is now known that

although the PPP and PPM families have dissimilar sequence, the structure and mechanism of catalysis are exceedingly similar and one of the classical examples of convergent evolution.

PPP Family:

The PPP family of enzymes contributes to the major portion of serine/threonine phosphatase activity present in cells both because of large number of members in this family and also the high levels of expression in the cell. All members of the PPP group of enzymes share a common catalytic core domain of ~280 amino acids that are highly conserved across phyla. Indeed, some members of the PPP family such as PP1 and PP2A have sequence similarity approaching nearly 97% between humans and *C. elegans.*PPP members are among some of the most highly conserved proteins in evolution(Kennelly 2001).

The PPP family commonly contains atleast 7 classes of phosphatases, PP1 - 7 (Table 2) and a few more idiosyncratic atypical members in some species such as S. cerevisiae(Cohen 1997). As discussed above, most of the PPP family members utilize a modular design to achieve substrate specificity and fidelity. Regulatory subunits have been reported for almost every member of the PPP family with the exception of PP5 and PP7 (Andreeva and Kutuzov 1999). The PP5 is peculiar in that, apart from the core catalytic domain it also has additional (TPR) regulatory domains as part of the same protein that modulates its activity(Moorhead, Trinkle-Mulcahy et al. 2007; Moorhead, De Wever et al. 2009).

The PP1 (PPP1) family of enzymes is one of the most well known and well studied members of the PPP family. In humans, there are 3 members of the PP1 family (α,β/δ,γ) which are highly homologous to each other. In C. elegans, there exists 3 PP1 members of high similarity but this family has further expanded and there are an additional 40 PP1-like enzymes, with moderate sequence similarity to the PP1 catalytic subunit, whose functions are currently unknown. PP1 phosphatases are among the most versatile group of enzymes acting on diverse substrates such as glycogen, myosin, cell cycle kinases and other protein substrates. PP1 enzymes are modular and substrate targeting is performed by regulatory subunits. By

sequence analysis of known PP1 regulatory 'subunits' it was identified that the PP1 catalytic subunit is recruited using a relatively small docking motif, the RVXF/W motif. To date more than a 100 PP1 regulatory subunits have been described which mediate diverse functions from glycogen breakdown, cell-cycle progression, translational regulation and muscle contraction(Llorian, Beullens et al. 2004; Guergnon, Dessauge et al. 2006; Kwiek, Thacker et al. 2006; Pasder, Shpungin et al. 2006; Trinkle-Mulcahy 2006).

The PP2A (PPP2) phosphatase functions in the cell as a heterotrimeric modular enzyme. In contrast to PP1 subunits, which are quite variable, PP2A subunits are more easily recognized owing to sequence and structural similarities of different groups. The PP2A heterotrimeric holoenzmye consists of a structural subunit (A), the catalytic subunit (C) and a variable regulatory subunit (C) which determines the substrate specificity, sub-cellular localization and activity of the enzyme complex. In addition to association with regulatory subunits PP2A is also subject to post-translational modifications (C-terminal methylation and phosphorylation) that regulate its activity (Van Hoof and Goris 2004; Prickett and Brautigan 2006; Yoo, Boylan et al. 2007; Eichhorn, Creyghton et al. 2008; Janssens, Longin et al. 2008; Yan, Lavin et al. 2008; Shi 2009). Furthermore, some of the B-subunits themselves are subject to phosphorylation that might modulate their association/localization with the PP2A A-C core dimer and therefore regulate the holoenzmye activity. Humans have 2 Phosphatase catalytic subunits (PPPα ,β), 2 structural (A) subunits (PR65 α,β) and atleast 16 members in separate families of regulatory (B) subunits (B55, B56, B72 and striatin). The *C. elegans* genome contains a single catalytic (C) and structural subunits (A) and 7 regulatory subunits (B).

PP2A is known to be involved in the regulation of metabolism, mitosis, cell survival, and variety of other essential cellular processes but its function as a potential tumor suppressor has garnered the most attention. Mutations in the structural subunit that interferes with the binding of catalytic or regulatory subunits have been linked to several tumors(Xu, Xing et al. 2006). Similarly, N-terminal deletions of B56

subunits were shown to be correlated with higher metastatic potential in melanomas and complete loss of B56 subunits were seen in lung-cancer cell lines. Re-expression of the B56γ subunit in these cells resulted in a partial reversal of the cancer phenotype. Many natural tumorigenic toxins and viruses target the PP2A enzyme. For instance, the SV40 small T-antigen associates with the PP2A core dimer by mimicking a B-type regulatory subunit. It was shown that this interaction reduced the PP2A activity and thereby brought about cellular transformation. Indeed knocking down B56γ was sufficient to replicate the tumorigenic effects of SV40 small T-antigen(Chen, Possemato et al. 2004; Van Hoof and Goris 2004). The marine toxin, Okadaic acid, also effects its tumor promoting activities by reducing PP2A activity. The PP4 and PP6 are closely related structurally and functionally to PP2A. They both utilize regulatory subunits to target their substrates.

PP2B (PPP3) or calcineurin is a heterodimeric enzyme composed of a catalytic and a regulatory subunit (calmodulin-related). PP2B has an autoinhibitory C-terminal sequence that upon binding calcium moves away from the active site and stimulates activity of the enzyme. PP2B/calcineurin is best known for their action in T-cell response where they activate NFATc by dephosphorylation. Active NFATc is required for T-cell maturation and therefore PP2B inhibitors like cyclosporine and tacrolimus are used clinically as immunosuppressants. PP2B activity has also been appreciated to be important in memory formation and beta cell function(Yamashita, Katsumata et al. 2000; Heit 2007). The PPP5 and PPP7 are functionally similar in that they both have regulatory domains incorporated near the catalytic domains (EF-hands for PPP7 and TPR-repeats for PPP5). Similar to PP2B, the repeats have auto-inhibitory function and allow activation of the enzyme only when certain conditions are met (protein interactions in the case of PPP5 and Calcium for PPP7)(Yang 2005). PPP5 is known to be nuclear and its activity might be important for DNA repair mechanisms, glucocorticoid and stress signaling pathways.

Classes and functions of Human protein phosphatases

Family	Class	Number of genes	Regulatory subunits	Example of function and/or (substrate)
Ser/ Thr phosphatases				
PPP family	PP1	3	>90 (e.g., Repo-Man)	Chromosome condensation
	PP2A	2	A, B‡, etc.§	Chromatid cohesion
	PP4	1	R1, R2, R3α/β, etc.¶	DNA repair (γ-H2AX)
	PP5	1	None	Cellular stress
	PP6	1	SAP1–3, etc.¶	NFκB pathway
	PP2B	3	Regulatory B, CaM	Immune response (NFAT)
	PP7	2	Unknown	
PPM family	PP2C	18	None	TGFβ signalling (SMADs)
PTP superfamily (CX_5R)				
Class I PTPs (classic*)	Receptor PTP	21		Cell adhesion/cytoskeletal
	Non-receptor PTP	17		Insulin signalling (insulin receptor)
Class I PTPs (DSPs)	MAPKP	11		MAPK signalling (MAPK)
	Slingshots	3		Actin dynamics (cofilin)
	PRLs	3		Unknown
	Atypical DSP	19		Mostly unknown (mRNA)
	CDC14	4		Cytokinesis, mitotic exit
	PTEN	5		PIP_3 phosphatase
	Myotubularins	16		PtdIns3P, PtdIns(3,5)P_2 phosphatase
Class II PTPs	CDC25s	3		Promotes mitosis (CDKs)
Class III PTPs	LMWPTP	1		Unknown
Asp-based catalysis (DXDXT/V)				
FCP/SCP family	FCP1	1	RAP74 of TFIIF	Transcription (Pol II)
	SCP	3		TGFβ signalling (SMADs)
	FCP/SCP-like**	4		Unknown
HAD family		5‡‡		Actin dynamics (cofilin)

*Note that the division of receptor and non-transmembrane (non-receptor) is not absolute as the use of alternative splicing and promoters can generate both forms from one gene[1]. ‡Note that the B class of PP2A subunits includes B, B′, B″ and B‴ (see main text). §Additional PP2A C catalytic subunit-binding proteins are known, including α4 (also known as IGBP1; Tap42 in yeast) and Tip41 (see main text). ¶Additional PP4- and PP6-interacting proteins are known, including α4 and TIP41 (see main text). **Additional members with FCP1-like catalytic domains include Dullard, HSPC129, TIMM50 and MGC10067. ‡‡Even though this is a large superfamily of enzymes, so far only chronophin and Eya1–4 have been shown to display protein phosphatase activity. CaM, calmodulin; CDK, cyclin-dependent kinase; DSP, dual-specificity phosphatase; Eya, Eyes Absent; FCP, TFIIF-associating C-terminal domain phosphatase; H2AX, histone 2AX; HAD, haloacid dehalogenase; LMWPTP, low-molecular-weight PTP; MAPK, mitogen-activated protein kinase; MAPKP, MAPK phosphatase; NFκB, nuclear factor κB; NFAT, nuclear factor of activated T-cells; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; Pol II, RNA polymerase II; PPM, protein phosphatase, Mg^{2+} or Mn^{2+} dependent; PPP, phosphoprotein phosphatase; PRL, phosphatase of regenerating liver; PtdIns3P, phosphatidylinositol-3-phosphate; PtdIns(3,5)P_2, phosphatidylinositol-3,5-bisphosphate; PTEN, phosphatase and tensin homologue; PTP, protein Tyr phosphatase; SAP, Sit4-associated protein; SCP, small C-terminal domain phosphatase; Tap42, 2A phosphatase-associated protein of 42 kDa; Tip41, Tap42-interacting protein; TGFβ, transforming growth factor-β.

Moorhead *et al. Nature Reviews Molecular Cell Biology* **8**, 234–244 (March 2007) | doi:10.1038/nrm2126

Table 2

PPM family:

The PPM family members were distinguished initially by their requirement for exogenous divalent cations (Mg^{2+}, Mn^{2+}) and insensitivity to classical phosphatase inhibitors like Okadaic acid and Calyculin A(Bellinzoni, Wehenkel et al. 2007; Wehenkel, Bellinzoni et al. 2007) but sensitive to metal chelators like EDTA. Compared to the PPP family members the PPM members show more structural diversity. The N-terminal catalytic domain is similar in these enzymes but the C-terminal sequences are divergent which probably are responsible for the diverse array of functions by the PPM members. In contrast to the PPP family members, which usually recruit regulatory proteins, the PPM family of phosphatases generally acts as monomers.

In Humans there are atleast 18 members of this family including the metabolic enzyme Pyruvate dehydrogenase phosphatase (PDP) and PHLPP domain phosphatases(Lu and Wang 2008). The PP2C family members have also been known to function in regulation of the ras/MAPK pathway and TGFβ signaling (Chen, Shen et al. 2006). In addition, the PHLPP and PP2C isoforms were shown to directly regulate insulin/ IGF-1 growth factor signaling, DNA-damage response and apoptosis (Lu and Wang 2008).

Screen for Serine/threonine phosphatases modulating IIS:

Downstream of the Insulin/IGF-1 receptor the vast majority of the signaling occurs by regulatory modifications at serine/threonine residues. Consequently, regulation of the IIS pathway by serine/threonine protein kinases has been extensively studied. Serine/threonine phosphatases are important regulators for reverse reaction and to restore the original status of proteins. Phosphorylation can be either activating or inactivating depending on the context in the protein. For example, specific serine/threonine phosphorylations in the activation domain of kinases such as PDK1, AKT1/2/3 and SGK results in increased signaling activity in the cascade. However, S/T phosphorylation at specific sites in IRS proteins can negatively regulate signaling by making them poor substrates to the insulin receptor(Gual, Le Marchand-Brustel et al. 2005; Sun and Liu 2009). Similarly, FOXO can undergo both activation (JNK,MST1) and repression (AKT/SGK) by phosphorylation. (Wang, Bohmann et al. 2003; Oh, Mukhopadhyay et al. 2005; Lehtinen, Yuan et al. 2006)

In *C. elegans*, the lipid phosphatase DAF-18 (homologous to mammalian Phosphatase and Tensin Homolog, PTEN), was the only phosphatase known to function in the IIS pathway (Ogg and Ruvkun 1998; Gil, Malone Link et al. 1999; Mihaylova, Borland et al. 1999; Rouault, Kuwabara et al. 1999). However, little was known about the identity of protein serine/threonine phosphatases that regulate IIS pathway in *C. elegans*. To this end we performed a directed RNAi screen of serine/threonine protein phosphatases (PPP/PPM families) that affect phenotypes regulated by the IIS pathway.

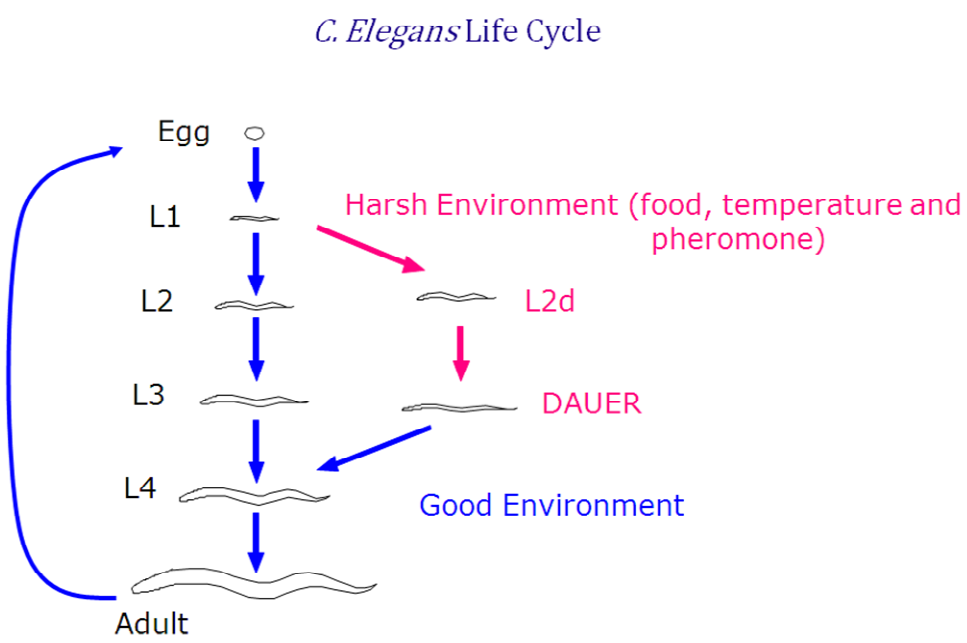
C. elegans development proceeds from an egg, through 4 larval stages into a self-fertilizing, hermaphrodite adult. However, under unfavorable growth conditions such as crowding and low food availability worms at L1/2 stage can enter a stage of diapause known as dauer (Riddle D. 1997)(Figure 11A). The dauer stage is characterized by increased resistance to adverse environmental conditions and allows

the worms to survive until the return of favorable growth conditions, when eventually; they can resume development to form into reproductive adults. Genetic screens to identify genes mediating dauer formation have resulted in the identification of several pathways that control dauer diapauses (Figure 11B).

Two main parallel signaling cascades, the TGFβ and the IIS pathways, were found to regulate dauer by eventually converging on *daf-12* (steroid nuclear hormone receptor). The TGFβ pathway consists of daf-7 (TGFβ ligand), *daf-1, 4* (type I and II receptors), *daf-8, 14, 3* (SMADS) and *daf-5* (Sno/ski). Mutations in *daf-7, 1, 4, 8, 14* are daf-c and suppressed by mutations in *daf-3* and 5. However*, daf-3* and *5* mutations cannot suppress IIS mutants.

The other major parallel pathway to control dauer formation is the IIS pathway. Of the IIS components described previously, null mutations in *daf-2, age-1, pdk-1* or both *akt1;akt-2* results in a constitutive dauer phenotype that can be completely suppressed by *daf-16.* It has been previously reported that loss of function mutations of *daf-16* cannot suppress TGFβ daf-c mutations (such as *daf-7*), however, such experiments used point mutants of *daf-16 (m26, m27*) and may not be valid. Most daf-c mutations in both IIS and TGFβ pathways can be suppressed by the daf-d mutation in *daf-12* indicating that both these pathways converge on *daf-12* for dauer formation(Albert and Riddle 1988; Riddle and Albert 1997).However, certain exceptions exist, certain strong alleles of *daf-2* (including *e1370*) are not suppressed by *daf-12* (lf) mutations and result in embryonic or intermediate larval arrest but these alleles are completely suppressed by *daf-16* (lf). These findings indicate that, in at least these alleles, DAF-16 and DAF-12 mediate dauer formation in a parallel but antagonistic fashion. DAF-16 activity precludes the developmental progression but the absence of DAF-12 prevents dauer formation thereby arresting the larvae in an intermediate stage.

A



B Pathways regulating dauer formation in *C. elegans*

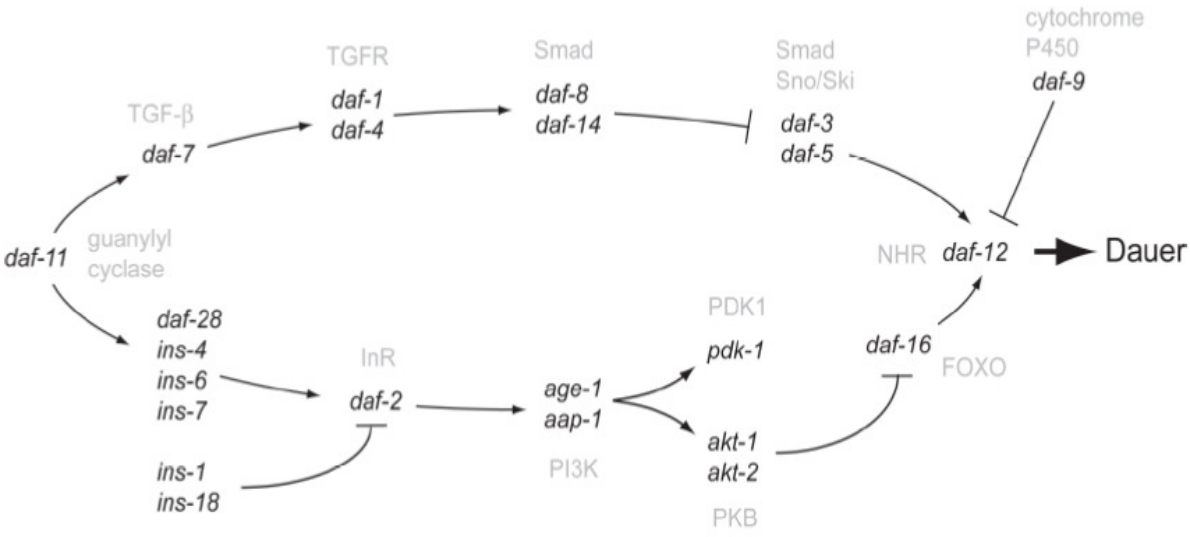


Figure 11

In order to identify phosphatases that regulate the IIS cascade in *C. elegans* we designed an RNAi modifier screen for dauer formation in *daf-2(e1370)* mutants. *daf-2(e1370)* is a temperature sensitive allele(Riddle, Swanson et al. 1981) and displays relatively normal growth (and no dauer formation) at 15^{o} C but arrests as 100% dauers at 25^{o}C(Kimura, Tissenbaum et al. 1997). In order to conduct the phosphatase RNAi screen, we decided to use an intermediate temperature of 20^{o}C for several reasons. Firstly, at 20^{o}C, partial signaling still exists in the pathway and it becomes possible to assay for both enhancers and suppressors of dauer formation. Serine/threonine phosphorylation is known to both activate and reduce signaling in the IIS pathway and therefore this strategy yielded candidates that both positively (increases dauer) and negatively (decreases dauer) regulate the IIS cascade upon RNAi knockdown. Secondly, since RNAi knockdown cannot usually mimic a complete loss of function it was necessary to use an intermediate temperature to increase the sensitivity of the screen.

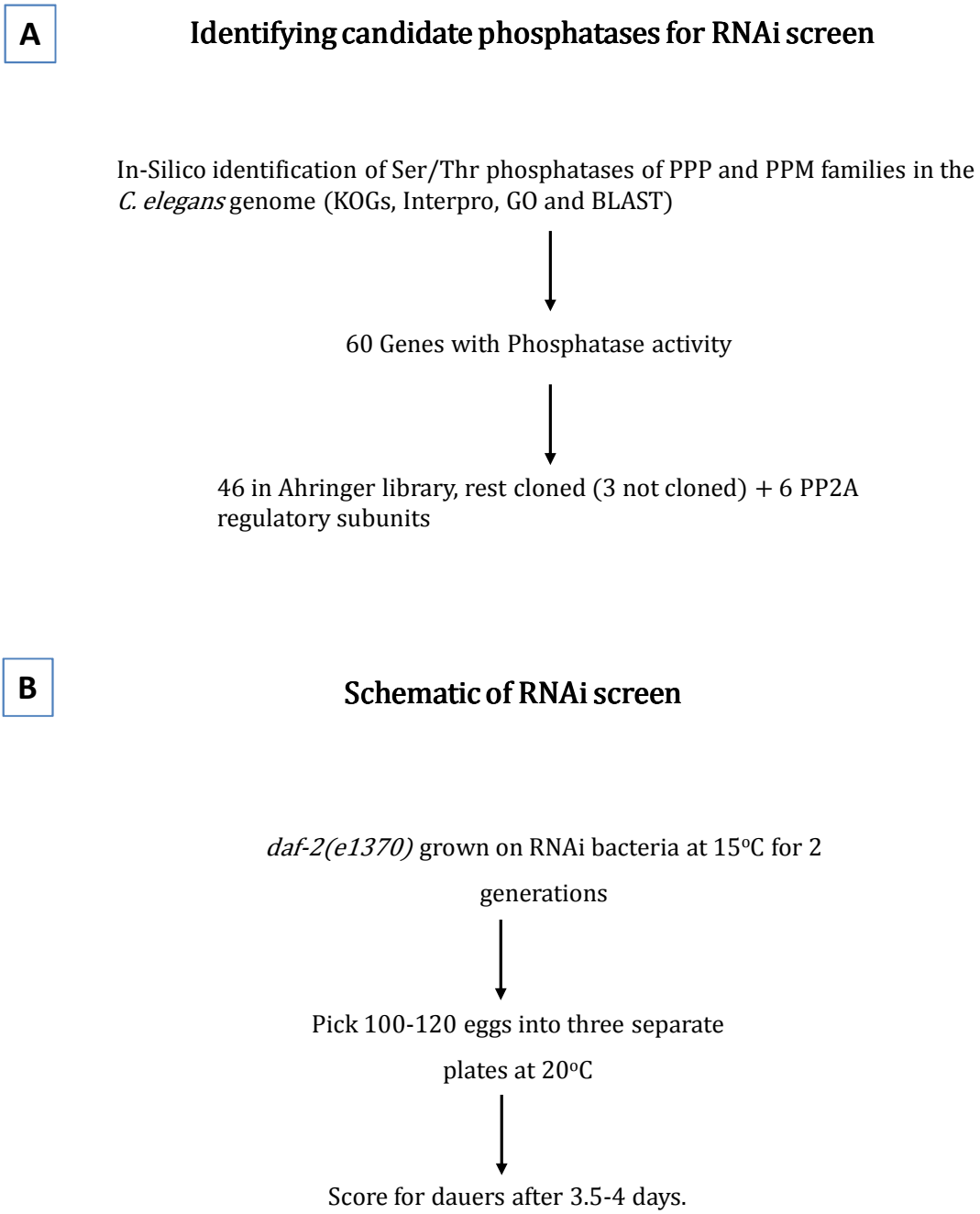

A
Identifying candidate phosphatases for RNAi screen
In-Silico identification of Ser/Thr phosphatases of PPP and PPM families in the C. elegans genome (KOGs, Interpro, GO and BLAST)
60 Genes with Phosphatase activity
46 in Ahringer library, rest cloned (3 not cloned) + 6 PP2A regulatory subunits
B
Schematic of RNAi screen
daf-2(e1370) grown on RNAi bacteria at 15°C for 2 generations
Pick 100-120 eggs into three separate plates at 20°C
Score for dauers after 3.5-4 days.

Figure 12

Results and Discussion

RNAi screen to identify phosphatases in IIS pathway

To identify the serine/threonine phosphatases in the *C. elegans* genome, we performed *in silico* analyses using both NCBI KOGs (clusters of euKaryotic Orthologous Groups) and WormBase (a *C. elegans* database: http://www.wormbase.org; WS152) annotations (Figure 12A). A total of 60 genes were identified for further analysis. We obtained RNAi clones for these phosphatases from the Ahringer RNAi library (Kamath and Ahringer 2003; Kamath, Fraser et al. 2003), generated them using available clones from the ORFeome library (Reboul, Vaglio et al. 2003) or cloned them *de-novo* using Gateway Technology (Invitrogen, USA; see Materials and Methods). We were unable to clone 3 of the phosphatase cDNAs and therefore screened a total of 57 candidates (Table 3).

In addition, we included 6 of the 7 annotated PP2A holoenzyme regulatory subunits (one was not cloned) in the screen for two reasons. First, a preliminary chemical inhibitor screen identified the PP2A family of phosphatases as important regulators of DAF-16 nuclear translocation (Padmanabhan and Tissenbaum, unpublished data). Second, RNAi of the catalytic (C) and structural (A) subunits of PP2A resulted in lethality (data not shown).

For the screen, *daf-2(e1370)* mutants were grown on RNAi-expressing bacteria for two generations, and eggs were picked onto 3 plates for each RNAi clone (Figure 12B). The plates were incubated at 20^{o}C and scored 3.5-4 days later for the presence of dauers and non-dauers. Since DAF-18 is the only known phosphatase that negatively regulates the IIS in *C. elegans*, we used *daf-18* RNAi as a positive control in all our experiments.

C. elegans Serine/Threonine phosphatase members of PPP, PPM family and PP2A regulatory subunits selected for RNAi screen

Phosphatase family	Members	*C. elegans* homologs
PPM	PP2C	10
	PDP	1
PPP	PP1	3
	PP2A	1
	PP2B	1
	PP4	2
	PP5	1
	PP6	1
	PP1 like	40
PP2A regulatory subunits	B55/PR55	1
	B56/PR61	2
	B72/PR72	4

Table 3

In order to compare potential candidates between successive experiments, percentage of dauers for a candidate was first calculated and then normalized by subtracting with the percent dauers in vector RNAi. This resulted in vector dauer% in each experiment to become zero and candidates that enhance dauer formation to have positive values and conversely candidates that suppress dauer to have negative values (Non-normalized numbers are also shown - Table 5). A cut-off value of 50% (2 fold) was chosen for both dauer suppressing and dauer enhancing candidates.

From a total of 63 RNAi clones (57 phosphatases and 6 PP2A regulatory subunits), we identified 4 phosphatases that decreased *daf-2(e1370)* dauer formation to a level s less than 50% of Vector RNAi and 16 phosphatases that increased *daf-2(e1370)* dauer formation to a level s more than 50% of Vector RNAi.(Table 4)

Candidates suppressing dauer formation:

Genes that reduced dauer formation in the *daf-2(e1370)* mutants, upon knockdown, were putative negative regulators of IIS (Table 4A). The small number of genes (4) that showed the dauer suppression phenotype indicated that this was likely a specific effect. Moreover, previous genetic studies, have consistently identified only components of IIS as suppressors of dauer formation in *daf-2(e1370)*.

daf-2 (lf) alleles can be classified into 2 main classes based on phenotypic pleiotropy and location of the mutation. In general, Class I alleles are a result of mutation in the ligand binding domain and show daf-c, age (lifespan increase) and thermotolerance phenotypes. Class II alleles show additional phenotypes apart from Class I like, embryonic, L1 arrest, slow movement, reduced brood size and high incidence of internal hatching all of which are suppressible by *daf-16*.

daf-2(e1370) worms, a class II allele, also has defects such as slow growth (apparent even at 15^{o}C), small brood size, internal hatching, stress-resistance and lifespan. Although all of these phenotypes are dependent on *daf-16,* it was possible that some phosphatases regulate only certain aspects of IIS and therefore affect only a subset of these phenotypes. Therefore, any apparent effects on other phenotypes like growth, brood size and internal hatching were also noted when appropriate.

A secondary screen was conducted with another *daf-2(e1368)* (Class I) allele to verify that the suppression of dauer formation was class independent. Candidates were further characterized in varying details as discussed below.

fem-2:

Our top candidate, *fem-2* (T19C3.4) is a PP2C phosphatase known to function in *C. elegans* sex determination (Pilgrim, McGregor et al. 1995; Hansen and Pilgrim 1998). The secondary screen with *daf-2(e1368)* revealed that *fem-2* could not suppress the dauer formation in this strain. This indicated that *fem-2* activity was specifically affecting *daf-2* dauer phenotype in a class II dependent manner. *fem-2* RNAi resulted in DAF-16::GFP being more cytosolic in a *daf-2(e1370);daf-16::gfp* strain (observation). The human homologue of fem-2, hFem-2 has been previously identified as a Ca^{2+}/Calmodulin dependent protein kinase phosphatase (CaMPKPase)(Tan, Chan et al. 2001). Moreover they also show that hFem-1 and *C. elegans* FEM-2 can mediate apoptosis in a caspase dependent manner. This effect was specific to FEM-2/hFem-2 and other PP2C enzymes did not cause apoptosis. CaMPKinases are activated by phosphorylation and known to play important role in survival following ischemic insults. This study however, did not attempt to investigate in detail the pathway responsible for *fem-2* over expression leading to apoptosis. IGF-1 signaling is known to promote survival and prevent caspase dependent cell death and it is possible that *fem-2* regulates the IIS cascade in a cell autonomous manner by fem-2 acting as a negative regulator in the IIS pathway through CAMPKinase.

Alternatively, CaMPK has also been shown to be important in insulin stimulated glucose transport probably at the level of vesicle trafficking. Inhibition of CAMPK results in reduction of insulin activated transport by more than 80%(Konstantopoulos, Marcuccio et al. 2007). Recent analysis of *daf-2(e1370)* and comparison with known mutations in the mammalian Insulin receptor indicates that insulin activated signaling through certain alleles of *daf-2* such as *e1370* and *sa223* can be overcome by using larger amounts of insulin(Patel, Garza-Garcia et al. 2008). Taken together with the known function of CAMPKinase in vesicle trafficking, *fem-2* RNAi could increase CAMPKinase activity resulting in an increase in the released quanta of insulin-like ligands in *C. elegans.* This in turn could result in suppression of dauer formation by reactivating IIS signaling in a cell non-autonomous manner. This mechanism would predict that *fem*-2 RNAi mediated dauer suppression will be lost in *daf-2* alleles with mutations in the ligand binding domain (for example*,e1368)* which agrees with the observed data. This hypothesis could be potentially tested by making *daf-2* double mutants with the *C. elegans* CAMPKinase homologue, *unc-43* and performing RNAi epistasis experiments for *fem-2* mediated dauer suppression.

***pptr-1/*PP2A regulatory subunits:**

The next dauer suppressor of *daf-2(e1370) dauer* in the screen was the B56 homologue, *pptr-1*. *pptr-1* RNAi suppressed the *daf-2(e1368)* strain equally well. An in-silico analysis of potential interactions using the tools developed by Zhong et al.(Zhong and Sternberg 2006) identified akt-2 as a predicted interaction partner extrapolated from data available in Drosophila Yeast 2 hybrid experiments. Zhong et al. used a novel method to identify functional interactors of any given gene by collectively utilizing interactome, expression, phenotype and functional annotation from *C. elegans, S. cerevisiae and D. melanogaster*. We found their method remarkably accurate for recapitulating known interactors. Subsequent genetic and biochemical characterization of this candidate revealed PPTR-1 to be responsible for dephosphorylating AKT-1 at the Thr-350 (PDK-1) site. (Please see the next chapter for a more detailed characterization of *pptr-1*.)

ZK973.3/Pyruvate dehydrogenase phosphatase(PDP):

The third candidate that suppressed dauer formation was ZK973.3/PDP (a PP2C family member). PDP has a known metabolic function in dephosphorylating and activating pyruvate dehydrogenase complex (PDC). PDC is a large multimeric mitochondrial matrix enzyme that is responsible for converting pyruvate to acetyl-CoA which is then oxidized in the mitochondria for ATP production. PDC is phosphorylated by the pyruvate dehydrogenase kinase (inhibitory) and activated after dephosphorylation by PDP. Although PDC is exclusively mitochondrial, PDP is also found in the cytosol and nucleus. PDP was recently shown to dephosphorylate SMAD in the TGFβ pathway(Chen, Shen et al. 2006). Further analysis of this gene showed that PDP RNAi can suppress *daf-2(e1368), daf-2(e1370);akt-1(ok535)* dauers and *daf-2(e1370)* lifespan and heat-stress (data not shown). These findings indicate that PDP RNAi can modulate most aspects of IIS. Further study of this gene is being carried out by another graduate student in our lab.

Candidates enhancing dauer formation:

Genes that enhance dauer formation in the *daf-2(e1370)* mutants, upon knockdown, are putative positive regulators of IIS (Table 4B). The results of the screen show a relatively large number of genes (16) showing significant enhancement of dauer formation which is not surprising given the complex nature of inputs into the dauer decision pathway. Known inputs into the dauer pathway are pheromone, temperature, food-signals and probably metabolic signals. Sensory neurons integrate environmental signals with internal cues to decide commitment to dauer formation. Candidates that enhance dauer formation upon knockdown could potentially act through several mechanisms.

First, they could act directly as a component in the IIS pathway. Second, act synergistically with other dauer formation pathways such as the TGFβ, daf-11, and steroid receptor pathways. And lastly, could be a non-specific reason of general slowing in development /growth (gro) as a result of knocking down an essential

gene. Furthermore, *daf-2 (e1370)* and other mutations in the IIS pathway can themselves result in a gro phenotype in a daf-16 dependent manner. The delay in development could potentially be misinterpreted as an increase in dauer formation because all plates were scored at the same time point. Usually all genes were subject to two generations of RNAi, unless it resulted in lethality or severe growth defects, to maximize knockdown and this usually resulted in uncovering of a gro phenotype. We do not find a consistent correlation between genes exhibiting gro/lethality and genes increasing dauer formation. For example, RNAi of W09C3.6, *sur-6* resulted in gro/embryonic lethal phenotypes but did not show dauer enhancement phenotypes. In contrast, the top dauer enhancing genes like *gsp-1, tax-6* showed gro phenotypes but were associated with more dramatic dauer enhancement.

Further characterization of this set of candidates was not done in this study. However, two candidates that enhance dauer formation have already been reported to act in the c. elegans IIS pathway from previous studies. *tax-6,* a gene encoding the calcineurin (PP2B) phosphatase, has been previously described to be important in mediating thermo and chemotaxis in worms(Kuhara, Inada et al. 2002). Sensory input is a major input into dauer formation and defective sensory perception could possibly enhance dauer formation in a cell non-autonomous way. Recently, however, *tax-6* (Dong, Venable et al. 2007) was identified on a large scale proteomic study as an important mediator and regulator of IIS in worms. *tax-6* is expressed in multiple cell types including neurons and the intestine. Similar to our results Dong et al. found that inhibiting *tax-6* increases dauer formation and extends lifespan of *daf-2* mutants in a daf-16 dependent manner. In addition, Lara-Pezzi, Winn et al have shown that a mammalian homologue and calcineurin variant, CnAβ, to inhibit foxo activity and promote muscle regeneration in mice. They also showed that phosphorylation of upstream kinases, such as PDK1 and AKT1 were compromised upon knock down of CnAβ (Lara-Pezzi, Winn et al. 2007). These findings indicate that the *tax-6* mediated regulation of IIS pathway may be conserved in worms and mammals.

Another candidate, F25D1.1 (PP2C), has been previously identified in two independent studies to be important in daf-16 mediated phenotypes (Wang and Kim 2003; Szewczyk, Udranszky et al. 2006). Wang et al. indentified F25D1.1 from microarray analysis of genes involved in dauer formation. They found that F25D1.1 levels were significantly downregulated in dauer larvae. In another independent transcriptome analysis, Szewczyk et al. identified F25D1.1 in the search of genes mediating lifespan and developmental delay in *C.elegans* maintenance medium (CeMM-a chemically defined liquid medium). Worms grown in CeMM show several phenotypes such as developmental delay and lifespan extension. They show that these phenotypes are a result of increased levels of *daf-16* expression. Under low signaling conditions DAF-16 is de-repressed from upstream signaling, allowing it to translocate into the nucleus and transactivate or repress downstream targets. Both studies show significant down regulation of F25D1.1 upon activation of DAF-16 and this taken together with our findings of enhanced dauer formation in the *daf-2(e1370)* mutant, suggests that F25D1.1 could be a potentially important downstream component of IIS/*daf-16* signaling. It would be interesting to identify if knocking down F25D1.1 can bypass the requirement of DAF-16 for *daf-16* dependent phenotypes. It is also possible that normal levels of F25D1.1 negatively regulate DAF-16 and knockdown of this candidate results in enhancing DAF-16 activity and increasing dauer formation. DAF-16 might itself negatively regulate F25D1.1 during low IIS conditions in order to maintain transcriptional activity and thus constitute a bistable negative feedback genetic switch.

Summary:

Phosphorylation is a critical and highly regulated post-translational modification in the cell. Kinases and phosphatases are both exquisitely regulated in order to balance the levels of signaling and to mediate intended responses. The insulin/IGF-1 signaling cascade is an evolutionarily conserved pathway that mediates important cellular and organismal responses such as survival, growth, metabolism, stress and lifespan and its dysregulation has implicated in diverse human diseases such as diabetes, cancer and dementia.

The insulin signaling pathway is known to be regulated by tyrosine and serine/threonine phosphorylations. The kinases and tyrosine phosphatases regulating the IIS pathway have been extensively studied but serine/threonine phosphatases have not been studied in such great detail due to the lack of appropriate reagents. In this study we designed an RNAi based screening strategy by utilizing a relatively well studied phenotypic output in worms. We utilized dauer formation as a good surrogate for the level of activity in the IIS pathway and this allowed us to screen a relatively large number of genes in a sensitive and quantitative fashion.

Our screen has identified several key positive and negative regulators of the *C. elegans* IIS pathway. We recovered known positive regulators of the IIS pathway in worms. *tax*-6, for example, has been shown to positively regulate IIS. We also identified important negative regulators in this pathway, one of which is *pptr-1*. We have further characterized *pptr*-1 and show, in the next chapter, that it acts at the level of *akt*-1 in IIS and is functionally conserved in regulating mammalian insulin signaling. Further work is needed to elucidate the mechanism of regulation of the insulin signaling cascade by these candidates and to verify if their homologues have similar conserved function in higher mammals.

Dauer modifier screen results

A

Top 4 candidates suppressing dauer formation by more than 50% of control

Gene	Common name	Family	Number of dauers	Total worms scored (N)	% dauers	% change relative to control RNAi	Std dev (+/-)
T19C3.8	fem-2	PP2C	0	388	0	-99	0
W08G11.4	pptr-1	PP2A-B56	7	411	2	-97	2
ZK973.3	ZK973.3	PP2C/PDP	32	366	9	-79	5
ZC477.2	ZC477.2	PP1-like (PP1γ)	70	286	25	-54	6

B

Top 16 candidates enhancing dauer formation by more than 50% of control

Gene	Common name	Family	Number of dauers	Total worms scored (N)	% dauers	% change relative to control RNAi	Std dev (+/-)
F29F11.6	gsp-1	PP1-like (PP1β)	135	137	99	459	1
C02F4.2	tax-6	PP2B	132	178	74	320	5
C23G10.1	C23G10.1	PP1-like	158	210	75	286	9
F25D1.1	tag-93	PP2C	171	278	62	248	6
F20D6.6	F20D6.6	PP1-like (PP1α)	132	276	48	170	1
T16G12.7	T16G12.7	PP1-like	124	207	60	159	19
W03D8.2	W03D8.2	PP1-like (PP1α)	136	309	44	148	13
C24H11.1	C24H11.1	PP1-like (PP1β)	95	169	56	143	16
Y75B8A.30	pph-4.1	PP4	101	107	94	128	14
R13A5.11	R13A5.11	PP1-like (PP1α)	177	412	43	120	21
C34D4.2	C34D4.2	PP1-like	165	332	50	115	10
F49E11.7	F49E11.7	PP1-like (PP1α)	181	379	48	106	9
C24H11.2	C24H11.2	PP1-like (PP1β)	82	180	46	97	5
F25B3.4	F25B3.4	PP1-like (PP1β)	94	280	34	89	5
C34C12.3	pph-6	PP6	132	162	82	84	9
F56C9.1	gsp-2	PP1-like (PP1α)	153	184	83	75	7

Table 4

Phosphatase screen results – individual experiments:

Table 5

Gene name	Number of dauers	Total(N)	%(dauers)	% change relative to control RNAi	Std dev (+/-)
Vector	155	326	48	0	1
Vector(Rpt)	152	322	47	0	3
daf-18	11	366	3	-44	1
C30A5.4	115	217	53	6	14
Y39B6A.2 (pph-5)	122	323	38	-10	13
F40E3.5	84	331	25	-22	1
Y54F10BM.1 (fbxa-1)	77	322	24	-23	7
Y49E10.3 (pph-4.2)	134	269	50	2	3
F23H11.8	144	350	41	-6	3
T03F1.5 (gsp-4)	151	337	45	-3	4
ZK938.1	198	366	54	7	6
F33A8.6	129	358	36	-11	9
F56C9.1 (gsp-2)	153	184	83	36	7
Vector	155	285	54	1	5
Vector(Rpt)	150	281	53	-1	5
daf-18	1	312	0	-54	1
T19C3.8 (fem-2)	0	388	0	-54	0
R08C7.8	114	205	56	2	10
ZC477.2	70	286	24	-29	6
C05A2.1 (pph-1)	102	246	41	-12	9
F26B1.5	92	215	43	-11	15
F22D6.9	138	315	44	-10	10
Y54G2A.24	119	250	48	-6	4
F42G9.1	87	234	37	-17	4
Vector	109	263	41	0	4
daf-18	3	338	1	-41	1
ZK973.3	32	366	9	-33	5
Y75B8A.30 (pph-4.1)	101	107	94	53	14
C47A4.3	86	346	25	-17	6
W09C3.6 (gsp-3)	70	295	24	-18	3
R08A2.2	116	228	51	9	9
R03D7.8	113	272	42	0	14
Vector	90	203	44	0	4
daf-18	0	319	0	-44	0
F52H3.6	116	216	54	9	10
C06A1.3	155	277	56	12	12

F58G1.3	153	238	64	20	7
C34C12.3 (pph-6)	132	162	81	37	9
F43C1.1	96	232	41	-3	8
Vector	62	318	20	0	3
daf-18	0	310	0	-20	0
R03D7.8	85	281	30	11	10
C23G10.1	158	210	75	56	9
R13A5.11	177	412	43	23	21
Vector	90	389	23	0	8
daf-18	0	362	0	-23	0
T23F11.1	114	317	36	13	21
T16G12.7	124	207	60	37	19
W09D10.4	56	366	15	-8	3
C24H11.1	95	169	56	33	16
C24H11.2	82	180	46	22	5
R08C7.8	30	308	10	-13	8
ZK354.9	79	380	21	-2	3
ZC477.2 (pph-5)	29	375	8	-15	5
C34D4.2	165	332	50	27	10
F42G8.8	95	356	27	4	1
C27B7.6	114	403	28	5	9
T25B9.2	100	389	26	3	4
Y69E1A.4	144	468	31	8	9
C42C1.2	81	434	19	-4	1
F49E11.7	181	379	48	25	9
Vector	71	405	18	0	4
daf-18	0	395	0	-18	0
C02F4.2 (tax-6)	132	178	74	56	5
W03D8.2	136	309	44	26	13
C47A4.3	22	216	10	-8	2
C09H5.7	70	280	25	7	9
F20D6.6	132	276	48	30	1
C01B7.6 (rpm-1)	59	327	18	0	9
F25B3.4	94	280	34	16	5
F25D1.1 (tag-93)	171	278	62	43	6
F29F11.6 (gsp-1)	135	137	99	81	1
C05A2.1 (fbxa-1)	60	396	15	-3	8
F23B12.1	104	391	27	9	2
Y40H4A.2	46	237	19	1	6
C44H4.5 (tap-1)	51	315	16	-2	6

(Table 5 Continued)

Acknowledgements for Chapter III:

A major portion of this section of the dissertation is taken from our paper:

Padmanabhan, S.*, Mukhopadhyay, A.*, Narasimhan, SD*., Tesz, G., Czech, M. P., and Tissenbaum, H. A. (2009). A PP2A regulatory subunit, PPTR-1, regulates *C. elegans* insulin signaling by modulating AKT-1 phosphorylation. Cell 136, 939-51

(*co-first author)

See Statement of Contribution for individual experiments

CHAPTER III

Characterization of PPTR-1

Abstract:

The *C. elegans* insulin/IGF-1 signaling (IIS) cascade plays a central role in the regulation of lifespan, dauer diapause, metabolism and stress response. The major regulatory control of IIS is through phosphorylation of its components by serine/threonine-specific protein kinases. In an RNAi screen for serine/threonine protein phosphatases that counteract the effect of the kinases in the IIS pathway, we identified *pptr-1*, a B56 regulatory subunit of the PP2A holoenzyme. Modulation of *pptr-1* affects phenotypes associated with the IIS pathway including life span, dauer, stress resistance and fat storage. We show that PPTR-1 functions by regulating worm AKT-1 phosphorylation at Thr 350. With striking conservation, mammalian B56β regulates Akt phosphorylation at Thr 308 in 3T3-L1 adipocytes. In *C. elegans*, this modulation ultimately leads to changes in subcellular localization and transcriptional activity of the forkhead transcription factor DAF-16. This work reveals a conserved role for the B56 regulatory subunit in modulating insulin signaling through AKT dephosphorylation and thereby has widespread implications in cancer and diabetes research.

Results

The second top candidate that suppressed dauer formation of *daf-2(e1370)* was *pptr-1*(Figure 13A). *pptr-1* (W08G11.4), is a member of the B56 family of genes encoding regulatory subunits of the PP2A protein phosphatase holoenzyme. The *C. elegans* genome contains 7 known PP2A regulatory subunit genes (*pptr-1* and *pptr-2*, B56 family; *sur-6,* B55 family; F47B8.3, C06G1.5, *rsa-1* and T22D1.5, B72 family; currently F47B8.3 is not annotated as a PP2A regulatory subunit according to WormBase Release WS200). To determine the specificity of *pptr-1* in regulating dauer formation, we re-tested the six PP2A regulatory subunits included in the screen for their ability to regulate dauer formation in *daf-2(e1370)*. As shown in Figure 13B, only *pptr-1* RNAi suppressed *daf-2(e1370)* dauer formation comparable to *daf-18* RNAi.

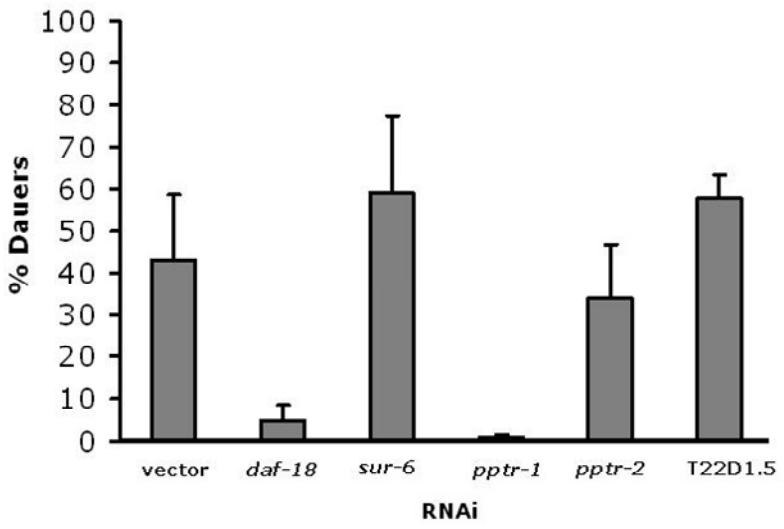
We next analyzed the effect of *pptr-1* RNAi on dauer formation of *daf-2(e1368)*. *pptr-1* RNAi significantly suppressed the dauer formation of *daf-2(e1368)* (84.6 ± 25.3 % on vector RNAi versus 19.8 ± 10.7 % on *pptr-1* RNAi; (Table 6). Therefore the effect of *pptr-1* RNAi on *daf-2* mutants is not allele-specific. Together these results indicate that *pptr-1* may function downstream of *daf-2.*

pptr-1 regulates dauer formation through the IIS pathway

To further investigate the role of *pptr-1* in dauer formation, we performed genetic epistasis analysis. In addition to the *C. elegans* IIS pathway, a second parallel TGF-β pathway also regulates dauer formation (Patterson and Padgett 2000; Savage-Dunn 2005). In this pathway, loss of function mutations in *daf-7* (TGF- β ligand), *daf-1* and *daf-4* (receptors) or *daf-14* and *daf-8* (R-Smads) lead to constitutive dauer formation; loss-of-function mutations in *daf-3* (Co-Smad) or *daf-5* (Sno/Ski) suppress these phenotypes (Ren, Lim et al. 1996; Patterson, Koweek et al. 1997; Gunther, Georgi et al. 2000; Inoue and Thomas 2000; da Graca, Zimmerman et al. 2004). However, null mutations in *daf-3* do not suppress *daf-2(e1370)* dauer

RNAi	% Dauer ± Std. Dev.	(n)
vector	17.5 ± 3.7	405
daf-18	0.0 ± 0.0	395
pptr-1	1.3 ± 1.6	470

A *pptr*-1, a regulatory subunit of the PP2A holoenzyme, was identified as a top candidate in a directed RNAi screen to identify serine/threonine phosphatases that regulate the IIS pathway.



B *pptr-1* is the only PP2A regulatory subunit family member that dramatically suppresses *daf-2(1370)* dauer formation. Error bars indicate the standard deviations among the different RNAi plates within one experiment.

Figure 13

formation (Vowels and Thomas 1992). Similarly, the current understanding is that, mutations in *daf-16* do not suppress *daf-7* dauer formation. In order to investigate if the role of *pptr-1* in dauer formation was exclusively through the insulin-signaling pathway we attempted to make a *daf-7(e1372);daf-16(mgdf50)* double. However, all recovered dauers were found to be wild-type for *daf-16(*data not shown). This indicated that daf-16 activity is required for *daf-7* dauer formation. To investigate the requirement of insulin signaling components in TGF-β pathway we knocked down *daf-16, daf-18* and *pptr-1 by RNAi in daf-7 (e1372)* worms (Table 6). All three RNAi vectors significantly suppressed *daf-7* dauer formation, with *daf-16* RNAi being most efficient (0% dauers at 25^{o}C). All previously published epistasis analysis were done with daf-16 point mutant (hypomorphic) alleles which were able to suppress *daf-2* dauer formation but probably had sufficient basal activity to allow the TGF-β pathway mutants to form dauers. In order to identify if the dauer suppression phenotype of *pptr-1* RNAi was because of its action in insulin/IGF-1 or the TGF-β pathway we decided to use an alternative approach.

In a *daf-2(e1370);daf-3(mgDf90)* double mutant, the input from the TGF- β pathway for dauer formation is essentially removed. In this strain, the dauer formation of *daf-2(e1370);daf-3(mgDf90)* worms was suppressed by *pptr-1* RNAi (87.6 ± 18.1 % dauers on vector RNAi to 28.4 ± 12.1. % dauers on *pptr-1* RNAi (Table 7); This data suggests that *pptr-1* controls dauer formation specifically through the IIS pathway and not the TGF-β pathway.

pptr-1 affects longevity, metabolism and stress response downstream of the IIS receptor

In addition to dauer formation, the *C. elegans* IIS pathway also regulates life span, fat storage and stress resistance (Barbieri, Bonafe et al. 2003; Mukhopadhyay, Oh et al. 2006). Since *pptr-1* regulates dauer formation specifically via the IIS pathway, we next determined whether this gene could also affect these other important phenotypes.

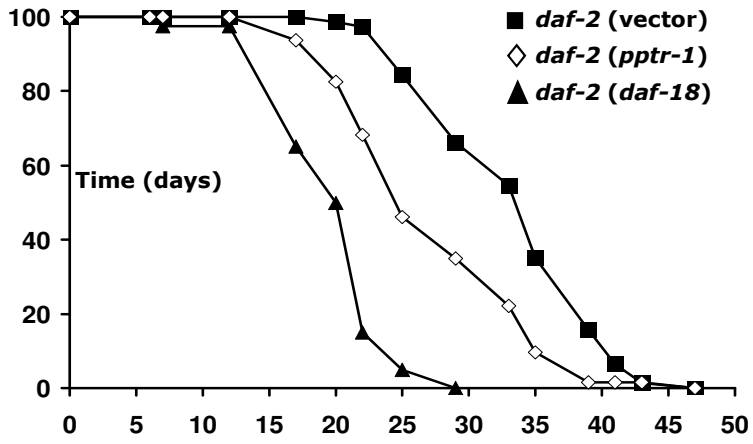
RNAi	%dauer± stddev	(n)
Vector	42±26	596
daf-18	21±2	345
pptr-1	7±3	490

Dauer formation in *daf-7(e1372)* worms upon RNAi. *daf-7(e1372)* on *daf-16* RNAi showed 0% dauers in an independent experiment.

Table 6

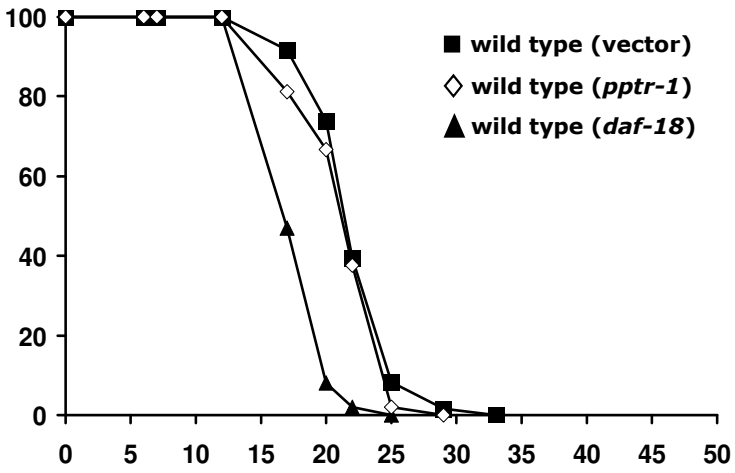
Mutations in *daf-2* result in life span extension (Kenyon, Chang et al. 1993) that is suppressed by loss-of-function mutations in *daf-18* (Dorman, Albinder et al. 1995; Mihaylova, Borland et al. 1999). To investigate whether *pptr-1* can regulate life span similar to *daf-18*, we determined whether knocking down *pptr-1* by RNAi could affect *daf-2(e1370)* life span. We grew wild type and *daf-2(e1370)* worms on vector, *daf-18* and *pptr-1* RNAi and measured their life span (Figure 14). Similar to *daf-18* RNAi, knock down of *pptr-1* resulted in a significant reduction in *daf-2(e1370)* life span (mean life span of *daf-2(e1370)* on vector RNAi is 33.9 ± 0.7 days , on *pptr-1* RNAi is 27.7 ± 0.9 days and on *daf-18* RNAi is 20.4 ± 0.6 days , p value < 0.0001; Figure 14). In contrast, lifespan of wild type worms was unaffected by *pptr-1* RNAi (mean lifespan of wild type on vector RNAi is 22.8 ± 0.4 days, is 21.9 ± 0.5 days on *pptr-1* RNAi and 18.6 ± 0.3 days on *daf-18* RNAi ; Figure 15). Thus, *pptr-1* affects phenotypes regulated by the IIS pathway, such as life span as well as dauer formation.

Life span extension correlates well with increased stress resistance (Lithgow and Walker 2002; Oh, Mukhopadhyay et al. 2005). For example, *daf-2(e1370)* mutants are not only long-lived but are also extremely resistant to various stresses such as heat and oxidative stress (Lithgow, White et al. 1995; Honda and Honda 1999; Munoz 2003). Therefore, we next analyzed the effect of *pptr-1* RNAi on the thermotolerance of *daf-2(e1370)* mutants. As anticipated, *pptr-1* RNAi also significantly reduced the thermotolerance of *daf-2(e1370)* mutants (on vector RNAi, *daf-2(e1370)* had a mean survival of 15.2 ± 0.7 hrs, whereas on *pptr-1* RNAi the survival was 13.8 ± 0.5 hrs (p value< 0.006). *pptr-1* RNAi did not affect the thermotolerance of wild type worms; mean thermotolerance was 9.8 ± 0.4 hrs on vector RNAi, versus 9.3 ± 0.3 hrs on *pptr-1* RNAi;Figure 16).



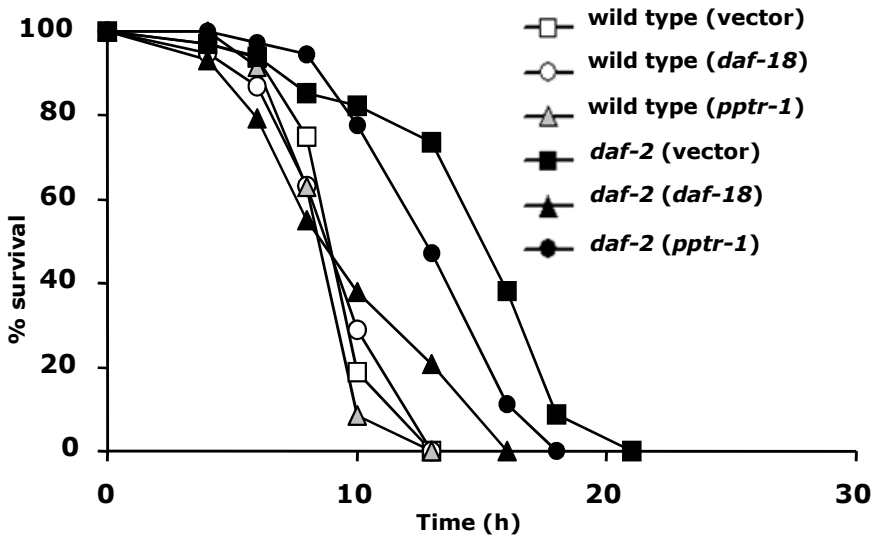
pptr-1 RNAi significantly reduces the life span of *daf-2(e1370)* mutants similar to *daf-18* RNAi (mean life on vector RNAi is 33.9 ± 0.7 days (n=77), *pptr*-1 RNAi is 27.7 ± 0.9 days (n=63) $p < 0.0001$and on *daf-18* RNAi is 20.4 ± 0.6 days (n=40), $p < 0.0001$)

Figure 14



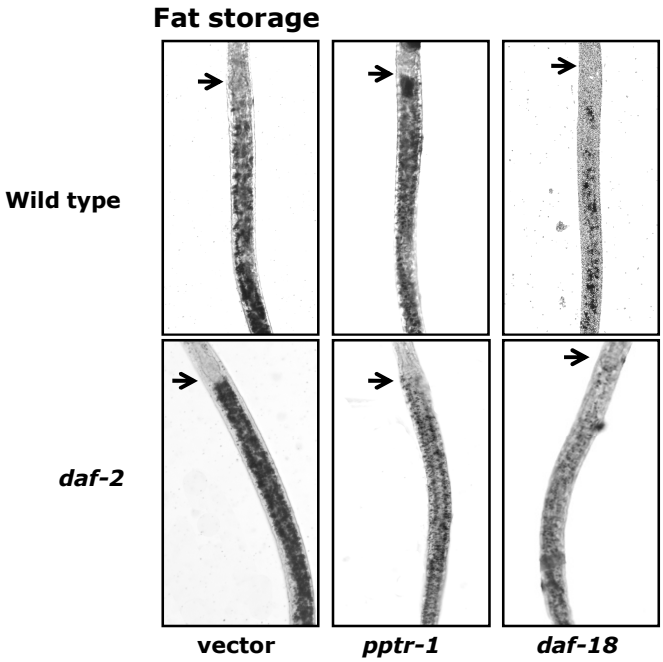
pptr-1 RNAi does not affect the life span of wild-type worms (mean life span on vector RNAi is 22.8 ± 0.4 days (n=61), *pptr*-1 RNAi is 21.9 ± 0.5 days (n=49). *daf-18* RNAi reduces mean life span of wild type worms to 18.6 ± 0.3 days (n=48) $p < 0.0001$).

Figure 15



Thermotolerance of *daf-2(e1370)* worms is reduced by *pptr-1* as well as *daf-18* RNAi (mean survival of *daf-2(e1370)* worms at 37°C on vector RNAi was 15.2 ± 0.7 hrs (n=34), whereas on *pptr-1* RNAi the survival was 13.8 ± 0.5 hrs (p value< 0.006) (n=36) and 10.3 ± 0.7 hrs (p value< 0.0001) (n=29) on *daf-18* RNAi. *pptr-1* RNAi did not affect the thermotolerance of wild type worms; (mean survival was 9.8 ± 0.4 hrs on vector RNAi (n=32), 9.3 ± 0.3 hrs on *pptr-1* RNAi (n=35) and 9.7 ± 0.4 hrs on *daf-18* RNAi (n=32).

Figure 16



Sudan black fat staining: *pptr-1* RNAi reduces the increased fat storage of *daf- 2(e1370)* worms, similar to *daf-18* RNAi but has no effect on wild type fat-storage. Arrows indicate the pharynx.

Figure 17

In addition to enhanced life span and stress resistance, *daf-2* mutants have increased fat storage (Kimura, Tissenbaum et al. 1997; Ashrafi, Chang et al. 2003). We next asked whether *pptr-1* could also affect fat storage in wild type and *daf-2(e1370)* worms using Sudan black staining. Consistent with our life span and stress resistance results, *pptr-1* RNAi suppressed the increased fat storage of *daf-2(e1370)* without affecting wild type fat storage (Figure 17). Together, these experiments suggest that *pptr-1* regulates multiple phenotypes associated with the IIS pathway in *C. elegans*.

pptr-1 functions at the level of *akt-1*

Signals from DAF-2 are transduced to the PI 3-kinase AGE-1 to activate the downstream serine/threonine kinase PDK-1 (Dorman, Albinder et al. 1995; Morris, Tissenbaum et al. 1996; Paradis, Ailion et al. 1999). PDK-1 in turn phosphorylates and activates three downstream serine/threonine kinases, AKT-1, AKT-2 and SGK-1 (Paradis and Ruvkun 1998; Hertweck, Gobel et al. 2004). These kinases together regulate the transcription factor DAF-16 by direct phosphorylation (Baumeister and Hertweck 2004). Mutations in *daf-16* suppress the enhanced dauer formation of *pdk-1* (Paradis, Ailion et al. 1999) or *akt-1*/*akt-2* mutants (Paradis and Ruvkun 1998; Oh, Mukhopadhyay et al. 2005). Thus far, our analysis suggests that *pptr-1* functions in the IIS pathway. We therefore sought to identify the potential target of *pptr-1* by performing genetic epistasis experiments.

First we analyzed the effect of *pptr-1* RNAi on dauer formation of the *pdk-1(sa680)* a putative null mutant. The dauer formation of *pdk-1(sa680)* was suppressed by *pptr-1* RNAi (95.6±1% dauers on vector RNAi versus 9.5 ± 0.3 % dauers on *pptr-1* RNAi at 25^{o}C (Table 7). *pdk-1 (sa680)* worms were sick on *daf-18* RNAi and had no effect on *pdk-1(sa680)* dauer formation or growth. Therefore, these results place *pptr-1* downstream of *pdk-1* and are consistent with the current understanding that *daf-18* acts upstream of *pdk-1*. Next, to investigate whether *pptr-1* acts at the level of *akt-1*, *akt-2* or *sgk-1*, we first analyzed dauer

Epistasis analysis of dauer formation using different IIS pathway mutants on RNAi.

Strains	% Dauer ± Std. Dev. (n)		
	vector RNAi	*daf-18* RNAi	*pptr-1* RNAi
daf-2(e1368)[a]	84.6 ± 25.3 (326)	0 (141)	19.8 ± 10.7 (104)
daf-2(e1370)	19.4 ± 2.7 (588)	1.9 ± 0.9 (528)	1.2 ± 1.0 (581)
daf-2(e1370);daf-3(mgDf90)	87.6 ± 18.1 (394)	41.6 ± 18.6 (345)	28.4 ± 12.1 (284)
pdk-1(sa680)[a]	95.6 ± 1.0 (490)	80.8[c] (52)	9.5 ± 0.3 (180)
daf-2(e1370)	13.5± 3.4 (221)	3.9± .1 (205)	0.9± 1.3 (186)
daf-2(e1370);akt-1(ok525)[d]	85.3± 5.8 (136)	10.5± 0.8 (349)	89.9± 1.6 (246)
daf-2(e1370);akt-2(ok393)	45.6± 6.7 (272)	8.0± 4.1 (311)	1.1± 0.8 (293)
daf-2(e1370);sgk-1(ok538)[d]	58.9± 3.6 (326)[e]	4.9± 2.8 (237)	1.5± 0.9 (210)
daf-2(1370);akt-1(ok525)[f]	82.1± 1 (459)	8.8(365)	84± 0.8 (540)

All strains were maintained at 15°C and assays were performed at 20°C, unless indicated otherwise. Also, dauer formation of all strains was scored after 3.5-4 days, unless indicated otherwise. Data shown is representative of one experiment.

[a] The experiment was performed at 25 °C

[b] Dauers were scored after 5 days.

[c] In most experiments, the *pdk-1(sa680)* worms failed to hatch on *daf-18* RNAi. This number represents 20% of the eggs picked for this assay.

[d] Dauers were scored after 7-8 days. For *daf-2(e1370);akt-1(ok525)* worms on vector or *pptr-1* RNAi, all the non-dauers were either partial dauers or dauer-like. They did not develop into adults even after 2 weeks.

[e] Dauers were scored after 7-8 days. The *daf-2(e1370);sgk-1(ok538)* strain shows a gro phenotype and worms remain at the L1/L2 stage for 6-7 days at 20°C.

[f] The *daf-2(e1370);akt-1(ok525)* at 19.2 °C

Table 7

formation in *akt-1(ok525)*, *akt-2(ok393)* and *sgk-1(ok538)* single mutants and the *akt-1(ok525);akt-2(ok393)* double mutant. While *akt-1(ok525)*, *akt-2(ok393)* and *sgk-1(ok538)* single mutants do not arrest as dauers at either 20 or 25 ^{o}C, the *akt-1(ok525);akt-2(ok393)* double mutant forms 100% dauers at all temperatures (Oh, Mukhopadhyay et al. 2005). To circumvent this problem, we generated double mutants of *daf-2(e1370)*;*akt-1(ok525), daf-2(e1370);akt-2(ok393)* and *daf-2(e1370);sgk-1(ok538)* and tested these strains for dauer formation on vector, *daf-18* and *pptr-1* RNAi. We reasoned that in a *daf-2* mutant background, the *akt-1, akt-2* and *sgk-1* mutants would exhibit temperature-induced dauer formation. Indeed, all three double mutants were able to form dauers at 20^{o}C. Importantly, *pptr-1* RNAi significantly suppressed dauer formation in *daf-2(e1370);akt-2(ok393)* (45.6± 6.7% dauers on vector RNAi versus 1.1± 0.8 % on *pptr-1* RNAi); In addition, *pptr-1* RNAi suppressed dauer formation of *daf-2(e1370);sgk-1(ok538)* worms (58.9± 3.6 % dauers on vector RNAi versus 1.5± 0.9 on *pptr-1* RNAi). In contrast, *pptr-1* RNAi did not affect dauer formation of *daf-2(e1370)*;*akt-1(ok525)* mutants (vector RNAi is 85.3± 5.8 % versus 89.9± 1.6 % on *pptr-1* RNAi). However, *daf-18* RNAi can suppress *daf-2(e1370) akt-1(ok525)* dauer formation (reduced to 10.5 ± 0.8%;) **Table 7**.

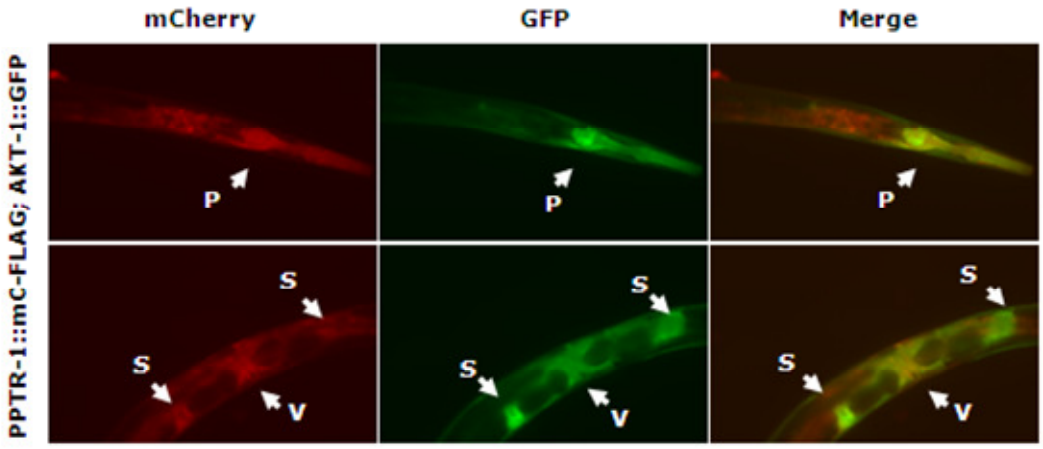
Although the dauer formation was not significantly different there was an important caveat to this experiment. The *daf-2(e1370) akt-1(ok525)* behaves differently from *daf-2(e1370)* alone by being enhanced for dauer formation at 20^{o}C. The non-dauer category in this experiment was fat and dark dauer-like animals with occasional pharyngeal pumping. These animals never exited this stage even after prolonged periods at 20^{o}C (plates were followed for more than 1 month). This raised a caveat that the RNAi phenotype of *pptr-1* was too weak to suppress dauer formation in this strain. Therefore we decided to perform the experiment at a lower temperature that allowed development to proceed but still allowed some worms to enter the dauer stage. We identified 19.2^{o}C as the best temperature for this experiment. We did not find any difference in dauer formation at this temperature between vector (82.1± 1 %) and *pptr-1* (84± 0.8 %)

indicating that in the absence of *akt-1, pptr-1* is unable to affect dauer formation in *daf-2(e1370)* mutants. These observations genetically place *pptr-1* at the level of *akt-1* in the IIS pathway.

PPTR-1 and AKT-1 are expressed in the same tissues

Since *pptr-1* and *akt-1* genetically interact, we wanted to investigate whether they have a common expression pattern. We generated or obtained *akt-1::gfp*, *akt-2::gfp*, *sgk-1::gfp* and *pptr-1::mC-flag* transgenic lines (see Materials and Methods; GFP refers to protein while *gfp* stands for transgene). We made double transgenic worms by crossing *pptr-1::mC-flag* worms to each of the above-mentioned GFP lines. Similar to published data, we observed AKT-1::GFP predominantly in the pharynx, several head neurons, the nerve ring, spermathecae and vulva (Paradis and Ruvkun 1998)(Figure 18); AKT-2::GFP in the pharynx (predominantly in the anterior region), somatic muscles, vulva muscles, spermathecae(Figure 19) (Paradis and Ruvkun 1998); SGK-1::GFP in amphid neurons, intestine and some tail neurons (Hertweck, Gobel et al. 2004) (Figure 20). PPTR-1::mC-FLAG was also observed in the pharynx, head neurons, nerve ring, spermathecae and vulva (Figure 18). To observe the sub-cellular localization of PPTR-1, we stained *pptr-1::mC-flag* worms with DAPI. We find that PPTR-1 is predominantly cytosolic with little DAPI overlap (Figure 21).

As shown in Figure 18(Merge), there is remarkable overlap between the expression patterns of PPTR-1 and AKT-1. We also observed partial overlap between AKT-2::GFP and PPTR-1::mC-FLAG, predominantly in the pharynx (Figure 19). SGK-1 and PPTR-1 are expressed in different tissues and we do not see any significant overlap (Figure 20).

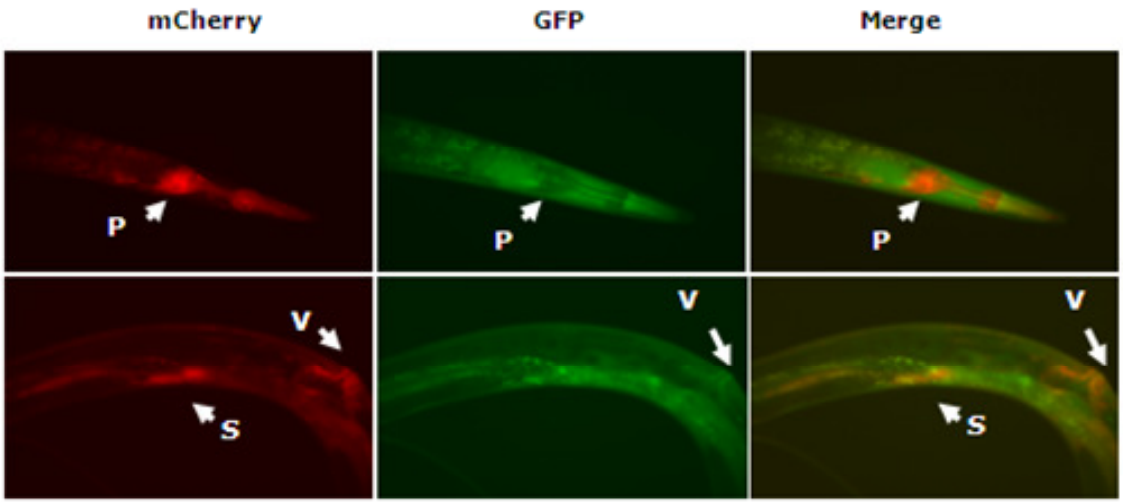


PPTR-1 co-localizes with AKT-1. *akt-1::gfp;pptr-1::mC-flag*, transgenic worms were mounted and visualized by fluorescence microscopy using Rhodamine (mCherry) and FITC (GFP) filters. PPTR-1 expression is observed mainly in the pharynx, vulva and spermatheca.

Expression of PPTR-1::mC-FLAG (mCherry) and AKT-1::GFP (GFP) in a *akt-1::gfp; pptr-1::mC-flag* strain.

Expression of PPTR-1::mC-FLAG completely overlaps with AKT-1::GFP (merge). Overlap is seen in pharynx, spermatheca, vulva, head neurons, tail neurons and coelomocytes.

Figure 18

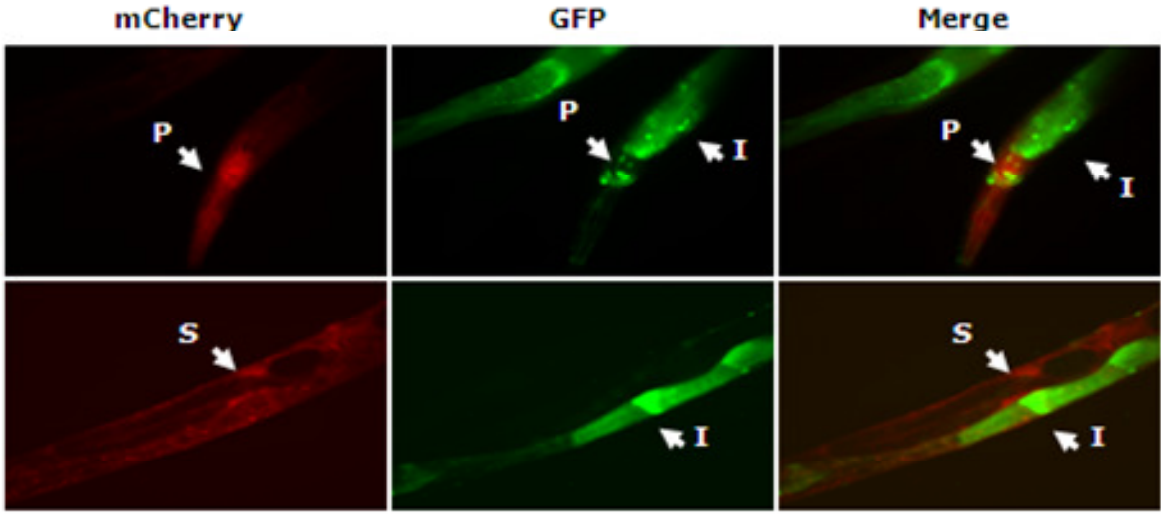


PPTR-1 partially co-localizes with AKT-2. *akt-2::gfp;pptr-1::mC-flag*, transgenic worms were mounted and visualized by fluorescence microscopy using Rhodamine (mCherry) and FITC (GFP) filters. PPTR-1 expression is observed mainly in the pharynx, vulva and spermatheca (A-C, mCherry).

Expression of PPTR-1::mC-FLAG (mCherry) and AKT-2::GFP (GFP) in a *akt-2::gfp; pptr-1::mC-flag* strain.

PPTR-1::mC-FLAG and AKT-2::GFP colocalize partially (in anterior pharynx, spermatheca and vulva). *akt-2::gfp (but not* PPTR-1::mC-FLAG *)* is present in somatic muscle and germline whereas *pptr-1::mCherry (*but not AKT-2::GFP) is present in head neurons and anterior pharynx.

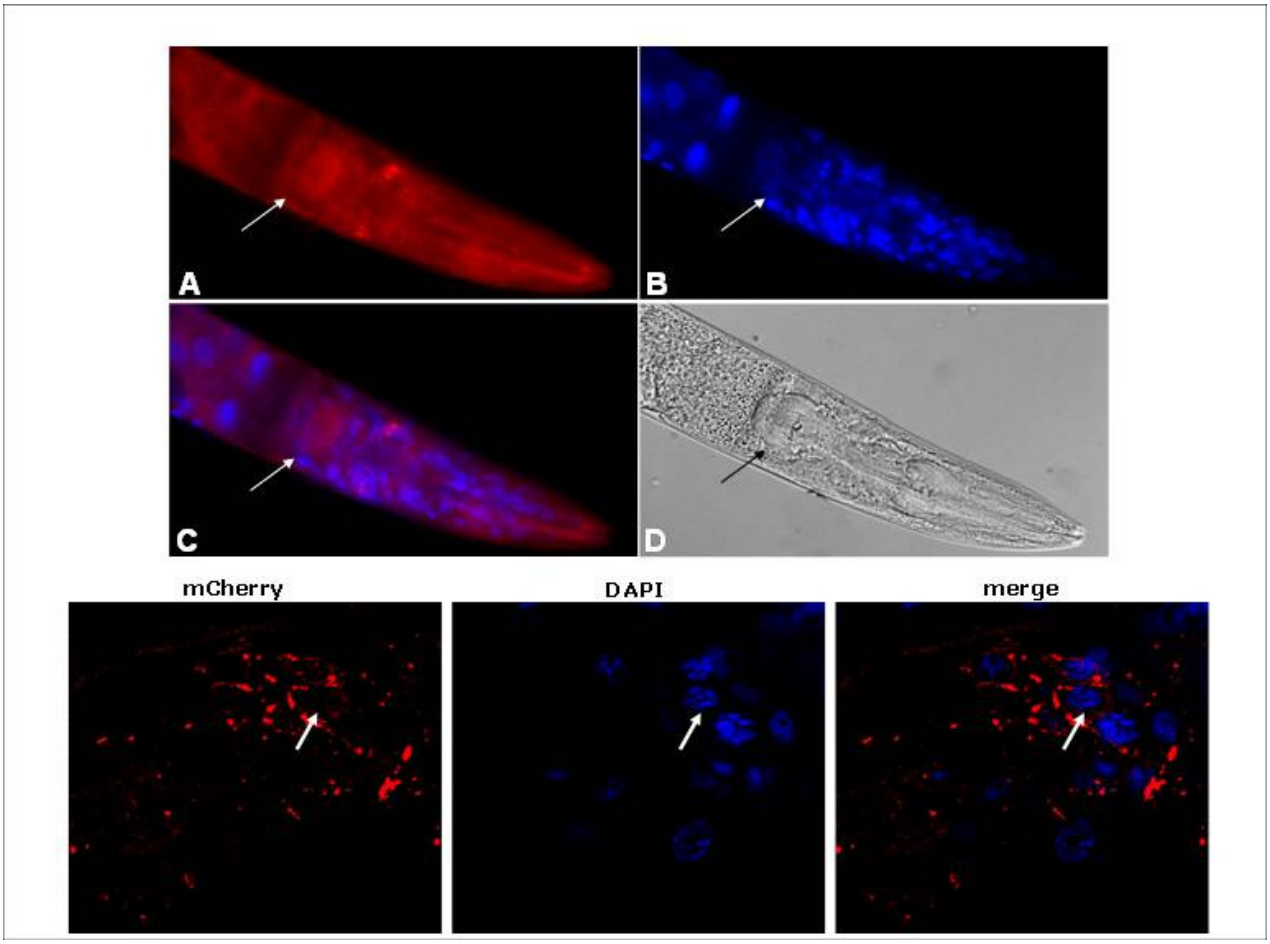
Figure 19



PPTR-1 does not co-localize with SGK-1. *sgk-1::gfp;pptr-1::mC-flag*, transgenic worms were mounted and visualized by fluorescence microscopy using Rhodamine (mCherry) and FITC (GFP) filters. PPTR-1 expression is observed mainly in the pharynx, vulva and spermatheca.

Expression of PPTR-1::mC-FLAG and SGK-1::GFP (merge) is completely different.

Figure 20



Upper panel: High resolution image of in *pptr-1::mC-flag* stained with DAPI at 600 X magnification. Arrow indicates the pharynx. (A) mCherry (B) DAPI (C) Merge (D) DIC.

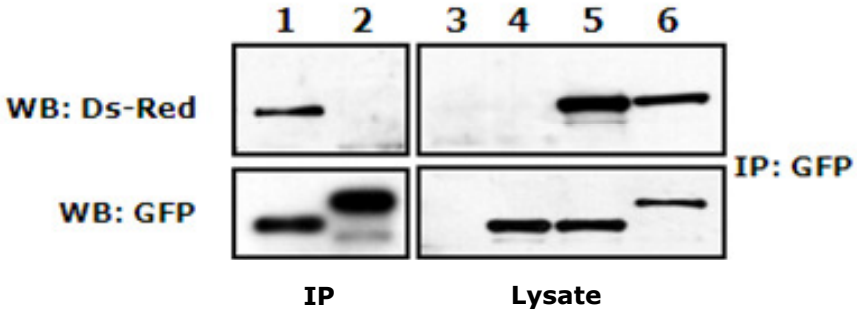
Lower panel: Subcellular localization of PPTR-1 as visualized in a single plane by spinning disk confocal microscopy after DAPI staining to delineate nuclei. Left, middle and right panels are mCherry at 561nm, DAPI at 405nm and the merge respectively

Figure 21

PPTR-1 regulates AKT-1 phosphorylation

Given the genetic epistasis as well as the overlapping expression patterns, we next determined whether PPTR-1 directly interacts with AKT-1 by co-immunoprecipitation (co-IP) in *C. elegans*. For all biochemical experiments, we used the PD4251 strain as a control. This strain contains *Pmyo-3::gfp* with a mitochondrial localization signal and *Pmyo-3::lacZ-gfp* with a nuclear localization signal (Fire, Xu et al. 1998). This strain will be referred to as *myo-3::gfp*. We prepared lysates from mixed-stage *akt-1::gfp; pptr-1::mC-flag* and *myo-3::gfp; pptr-1::mcherry-flag* transgenic worms. Following immunoprecipitation with either anti-FLAG or anti-GFP antibody, we found that PPTR-1 specifically interacts with AKT-1 and not with MYO-3::GFP (Figure 22). We also performed co-IP experiments to investigate whether PPTR-1 and AKT-2 interact, since we observed partial overlap in expression pattern of these proteins. We find that PPTR-1 does not interact with AKT-2. Although we find that PPTR-1::mC-FLAG and SGK-1::GFP interact in our co-IP experiments(Figure 23), our epistasis analyses show no genetic interaction between *pptr-1* and *sgk-1*. Moreover, we observe no overlap in the expression pattern of these two proteins using confocal microscopy. Hence, we do not believe this biochemical interaction to have a measurable functional output and did not pursue it further.

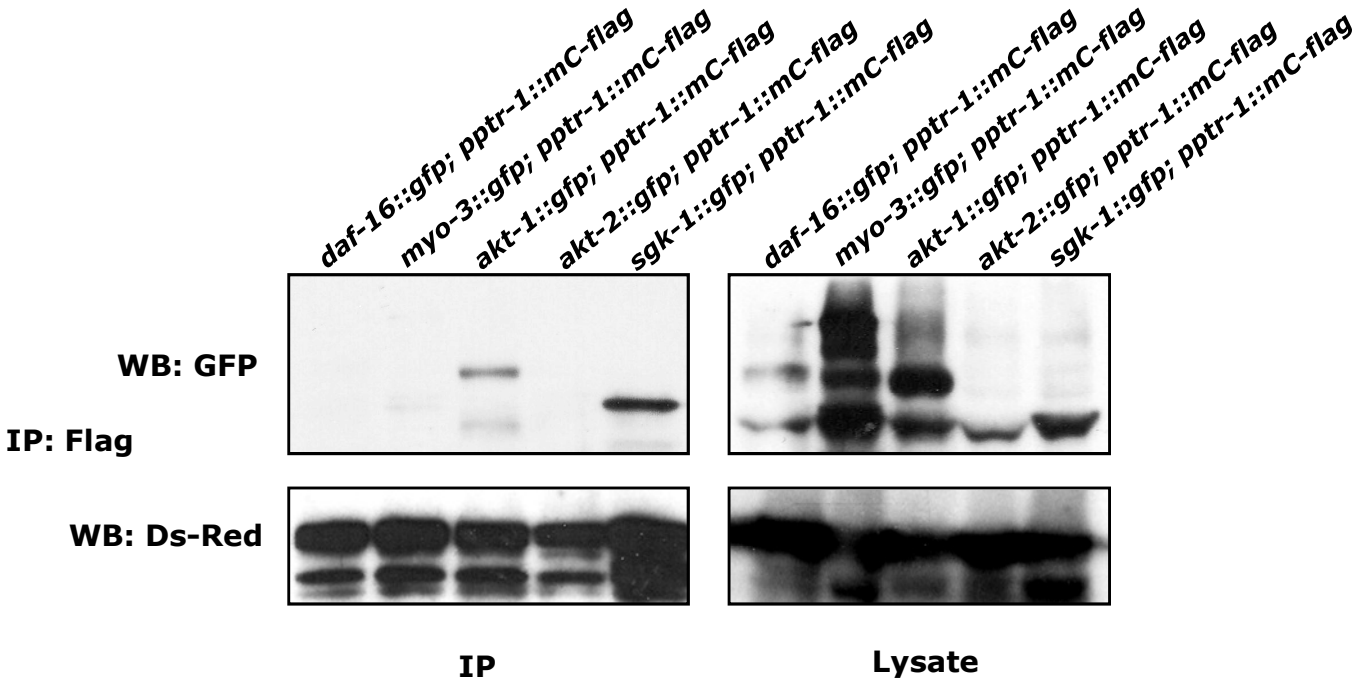
In mammals, Akt is activated by PDK phosphorylation at Thr 308 and PDK-2/TORC-2 protein complex at Ser 473 (Brazil and Hemmings 2001; Sarbassov, Guertin et al. 2005; Jacinto, Facchinetti et al. 2006). In *C. elegans* AKT-1, these sites correspond to Thr 350 and Ser 517, respectively. We generated affinity-purified phospho-specific antibodies (21st Century BioChemicals, USA; see Materials and Methods) against both sites to further investigate the role of PPTR-1 on AKT-1 phosphorylation.



PPTR-1 directly interacts with AKT-1 in *C. elegans*.

AKT-1::GFP and MYO-3::GFP were immunoprecipated (IP) from *akt-1::gfp;pptr-1::mC-flag(*1,5) and *myo-3::gfp;pptr-1::mC-flag(2,6)* strains using anti-GFP antibody and were analyzed by western blotting (WB) using anti-Ds-Red or anti-GFP antibodies. (3 – N2 lysate, 4-AKT-1::GFP alone control lysates)

Figure 22



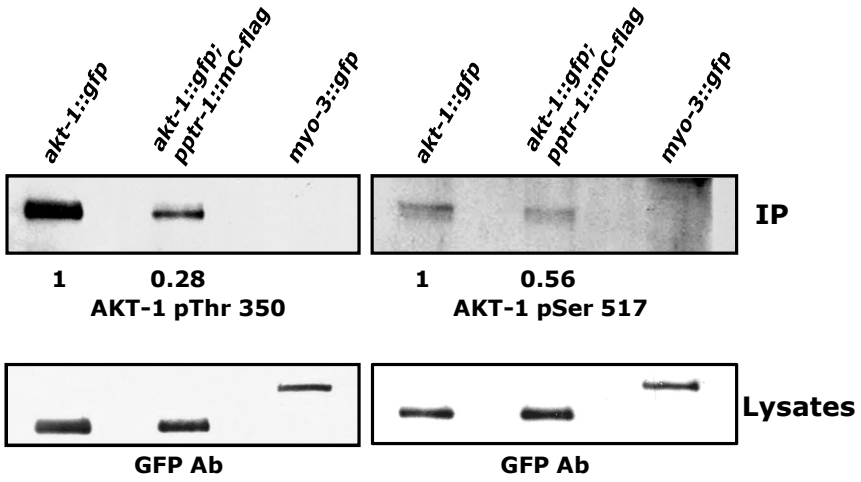
PPTR-1 directly interacts with AKT-1 in *C. elegans* but not with DAF-16, AKT-2 or GFP (control). We observe a biochemical interaction between PPTR-1 and SGK-1 in our co-IP experiments but do not see a genetic interaction or any tissue overlap between these proteins, PPTR-1::mC-FLAG was immunoprecipated (IP) from *akt-1::gfp;pptr-1::mC-flag*, *akt-2::gfp;pptr-1::mC-flag, sgk-1::gfp;pptr-1::mC-flag , daf-16::gfp;pptr-1::mC-flag* and *myo-3::gfp;pptr-1::mC-flag* using anti-FLAG antibody and interactions with AKT-1::GFP, AKT-2::GFP, SGK-1::GFP, DAF-16::GFP or MYO-3::GFP (control) and were analyzed by western blotting (WB) using anti-GFP or anti-Ds-Red antibodies.

Figure 23

Following immunoprecipitation with anti-GFP antibody from either *akt-1::gfp* or *akt-1::gfp;pptr-1::mC-flag* strain, we compared the phosphorylation status at these two sites. We find that overexpressing PPTR-1 can dramatically decrease the phosphorylation of the T350 site while having a marginal effect on the Ser 517 site (Figure 24). As a control experiment, we treated the immunoprecipitated AKT-1::GFP samples with lambda phosphatase and observed loss of the Thr and Ser phosphorylation, showing the specificity of the phospho-AKT antibodies (Figure 25). Thus, in *C. elegans*, PPTR-1 functions by directly regulating the dephosphorylation of AKT-1 primarily at the Thr 350 (mammalian Thr 308) site.

Mammalian PPTR-1 homolog regulates AKT-1 phosphorylation

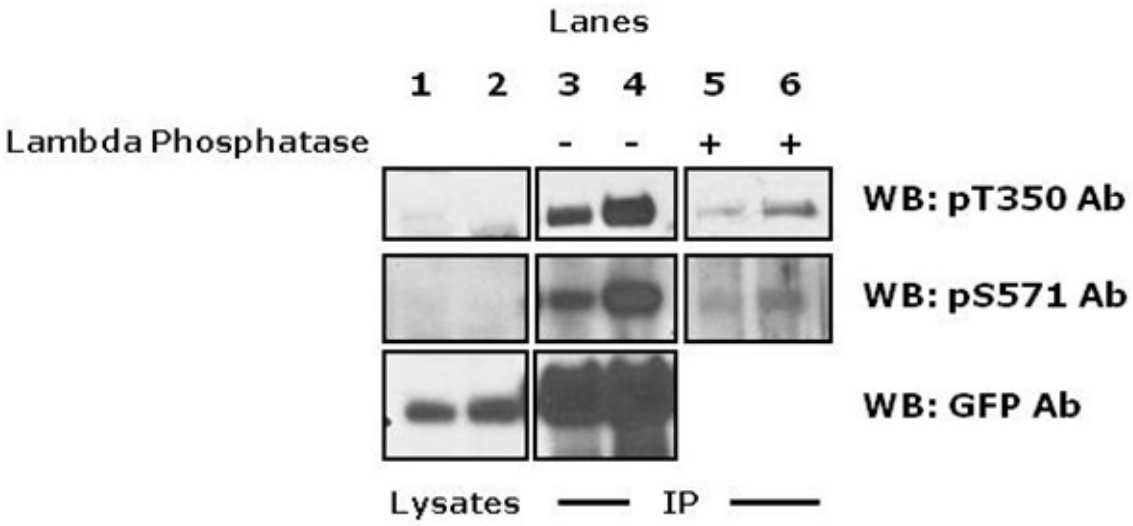
Given the evolutionary conservation of the *C. elegans* IIS pathway, we next determined if this mechanism of AKT-1 dephosphorylation mediated by PPTR-1 is also conserved in mammals. The mammalian B56 family of PP2A regulatory subunits has 8 members encoded by 5 genes that express in different tissues (Eichhorn, Creyghton et al. 2008). We used 3T3-L1 adipocytes to perform these studies since in this system, there is a well-characterized insulin signaling pathway that is responsive to changes in insulin levels (Ugi, Imamura et al. 2004; Watson, Kanzaki et al. 2004). We used microarray data comparing the expression profiles of fibroblasts to differentiated 3T3-L1 adipocytes (Powelka, Seth et al. 2006) to determine which B56 members were expressed in the adipocytes. We identified 2 genes, PPP2R5A (B56α) and PPP2R5B (B56β) as the top candidates. We knocked down either one or both these regulatory subunits by designing Smartpool siRNAs (Dharmacon, USA) and verified the silencing by quantitative RT PCR (Figure 27). Serum-starved siRNA-treated 3T3-L1 adipocytes were then stimulated with increasing concentrations of insulin. The cells were lysed and the proteins analyzed by western blotting using mammalian Akt phospho-specific antibodies (see Materials and Methods).



PPTR-1 overexpression reduces AKT-1 phosphorylation in C. elegans.

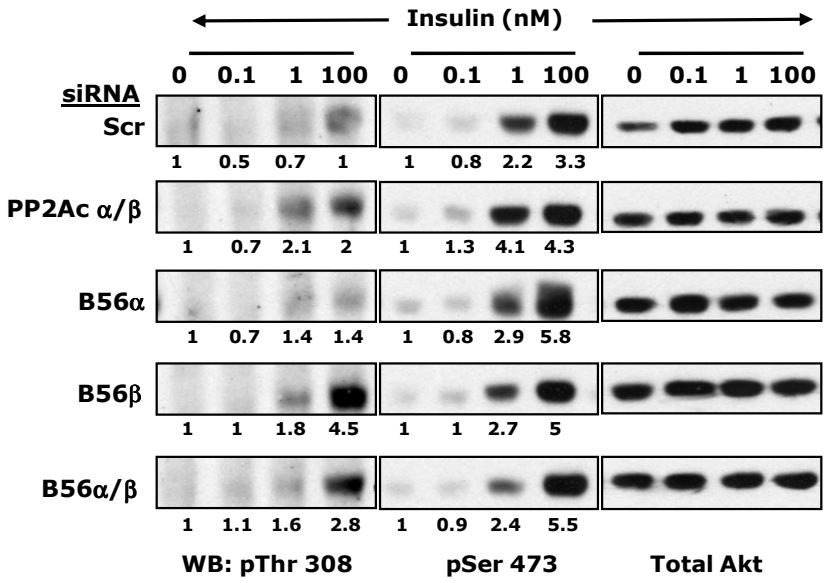
AKT-1::GFP and MYO-3::GFP were immunoprecipitated from akt-1::gfp, akt-1::gfp;pptr-1::mC-flag and myo-3::gfp;pptr-1::mC-flag followed by western blotting using pThr 350 or pSer 517 antibodies (upper panels). Total lysates were analyzed by western blotting (lower panels). Quantification of changes in AKT-1::GFP phosphorylation upon PPTR-1 overexpression is shown below each lane.

Figure 24



Determination of the specificity of the *C. elegans* phospho-AKT antibodies. L2/L3 (Lanes 1, 3 and 5) or mixed stage (Lanes 2, 4 and 6) *akt-1::gfp* worms were lysed and AKT-1::GFP was immunoprecipitated using anti-GFP antibody (Lanes 3-6). The immunoprecipitated proteins were resolved by SDS-PAGE and blotted to PVDF membrane. Lanes 5 and 6 of the membrane were cut out and treated with Lambda phosphatase. The treated as well as the untreated blots were probed with anti-phospho Thr 350 or Ser 517 antibodies.

Figure 25



Knock down of the mammalian B56β regulatory subunit by siRNA in 3T3-L1 adipocytes increases insulin-stimulated AKT phosphorylation at Thr 308.

3T3-L1 adipocytes were transfected with scrambled (Scr), PP2Acα/β, B56α, B56 β or B56α/β siRNA. These cells were then treated with increasing concentrations of insulin and phosphorylation status of Akt was analyzed by western blotting using pThr 308 (left) and pSer 473 antibodies (middle). Total Akt antibody was used as a loading control (right). Quantification of fold changes in Akt phosphorylation is shown below each lane.

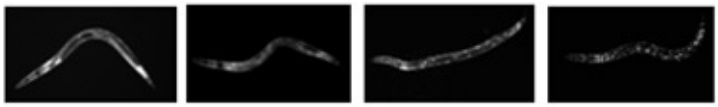
Figure 26

Knockdown of B56β results in a dramatic increase in phosphorylation at the Thr 308 site of Akt with relatively modest changes in Ser 473 phosphorylation(Figure 26). However, silencing of B56α had no effect on the phosphorylation status of Akt at either site. We observed that siRNA against both the PP2A catalytic subunits (PP2Acα/β) results in increased phosphorylation at Thr 308 but not at Ser 473. Together, these data suggest that PPTR-1/B56β subunit mediated regulation of IIS/AKT-1 is evolutionarily conserved.

PPTR-1 positively regulates DAF-16 nuclear localization and activity

DAF-16 nuclear localization

We next determined the consequences of modulating PPTR-1 dosage on the IIS pathway**.** In *C. elegans*, one of the major targets of AKT-1 is the forkhead transcription factor, DAF-16. Active signaling through the IIS pathway results in the phosphorylation of DAF-16 by AKT-1, AKT-2 and SGK-1, leading to its nuclear exclusion. However, under low signaling conditions, DAF-16 translocates into the nucleus, where it can directly bind and activate/repress the transcription of target genes involved in dauer formation, life span, stress resistance and fat storage (Oh, Mukhopadhyay et al. 2006). We asked whether *pptr-1* regulates IIS pathway-specific phenotypes by modulating DAF-16 function. Since we observed reduced phosphorylation of AKT-1 upon overexpression of PPTR-1, we first looked at the effect of PPTR-1 overexpression on DAF-16 nuclear localization (Henderson and Johnson 2001; Lee, Hench et al. 2001; Lin, Hsin et al. 2001). We generated a *daf-16::gfp;pptr-1::mC-flag* strain and compared the DAF-16 nuclear localization in these worms with a *daf-16*::*gfp* strain (*daf-16::gfp*, a kind gift from the Ruvkun lab). We categorized DAF-16::GFP localization as completely cytosolic, mostly cytosolic, mostly nuclear or completely nuclear. We find that DAF-16::GFP nuclear localization is enhanced when PPTR-1 is overexpressed (Table 8A).



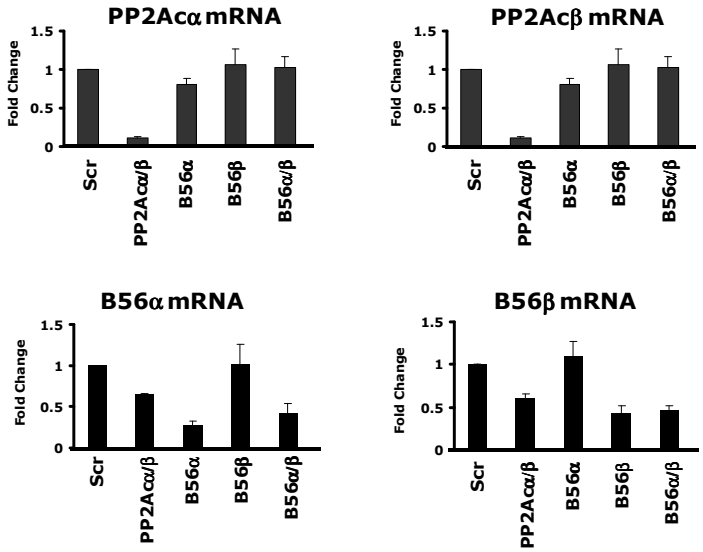
Strains	RNAi	Completely Cytoplasmic (%)	Mostly Cytoplasmic (%)	Mostly Nuclear (%)	Completely Nuclear (%)	(n)
pptr-1::mC-flag; daf-16::gfp	vector	34.5	22.9	29.6	12.9	77
	mCherry	79.5	18.6	1.95	0	96
daf-16::gfp	vector	50.0	42.5	5.0	2.5	40
	mCherry	55.3	29.8	14.9	0	47

A) Over-expression of PPTR-1 promotes DAF-16 nuclear translocation. On vector RNAi, DAF-16 is more enriched in the nucleus in a *pptr-1::mC-flag;daf-16::gfp* strain, compared to a *daf-16::gfp* strain. This effect is specific to the functional transgene, as knocking down *pptr-1::mC-flag* with *mCherry* RNAi decreases the extent of nuclear DAF-16.

Strain	RNAi	Completely Cytoplasmic (%)	Mostly cytoplasmic (%)	Mostly Nuclear (%)	Completely Nuclear (%)	(n)
daf-(e1370); daf-16::gfp	vector	4.5	40.9	50.0	4.5	22
	daf-18	63.6	45.4	4.5	0	25
	pptr-1	50.0	45.4	4.5	0	22

B) In a *daf-2(e1370);daf-16::gfp* strain, DAF-16 is enriched in the nucleus on vector RNAi, whereas on *pptr-1* RNAi as well as *daf-18* RNAi, DAF-16 is mostly cytosolic.

Table 8



Determination of specificity of siRNA knockdown.

The 3T3-L1 adipocytes were transfected with scrambled (Scr), PP2Aca/b, B56a, B56 b or B56a/b siRNA (indicated at the bottom of each graph). Total RNA was isolated from the adipocytes and quantitative RT-PCR analysis was performed to determine the efficiency of knock down of each gene (indicated at the top of each graph).

Figure 27

Strain	RNAi	High (%)	Medium (%)	Low (%)	(n)
daf-2(e1370); sod-3::gfp	vector	36.6	46.6	16.6	30
	daf-18	10.0	50.0	40.0	30
	pptr-1	6.6	43.3	50.0	30

A) *pptr-1* RNAi affects DAF-16 transcriptional activity. *sod-3* is one of the direct targets of DAF-16. *pptr*-1 RNAi reduces *Psod-3::*GFP expression in a *daf-2(e1370);Psod-3*::*gfp(muIs84)* strain, similar to *daf-18* RNAi.

Strain	RNAi	Transcripts determined by Q-PCR (% of vector RNAi)			
		sod-3	*hsp-12.6*	*sip-1*	*mtl-1*
daf-2(e1370)	*daf-16*	7.8	10.8	24.7	0.7
	daf-18	46.8	35.2	84.1	17.8
	pptr-1	45.2	52.2	86.7	32.0

B)Transcript abundance of known DAF-16 target genes decrease when *daf-2(e1370)* worms are grown on *pptr-1* RNAi, similar to *daf-18* RNAi, as detected by real-time PCR

Table 9

To determine the specificity of this response, we used *mCherry* RNAi to effectively knock down *mCherry* expression in *pptr-1::mC-flag* thereby reducing the expression levels of *pptr-1* transgene . Our results show that the enhanced nuclear localization upon PPTR-1 overexpression is suppressed when *pptr-1::mC-flag;daf-16*::*gfp* worms are grown on *mCherry* RNAi (Table 8A). However, *mCherry* RNAi has little effect on DAF-16 localization in *daf-16*::*gfp* worms. These experiments suggest that increased dosage of *pptr-1* affects DAF-16 nuclear localization. Consistent with its role in the *C. elegans* IIS pathway, we find that overexpression of *pptr-1* significantly increases the life span of wild type worms (Figure 28); mean life span of wild type is 23.9 ± 0.3 days, *pptr-1::mC-flag* is 30.1 ± 0.5 days, p<.0001, and the *unc-119(+); unc-119(ed3)* control strain is 22.6 ± 0.3 days).

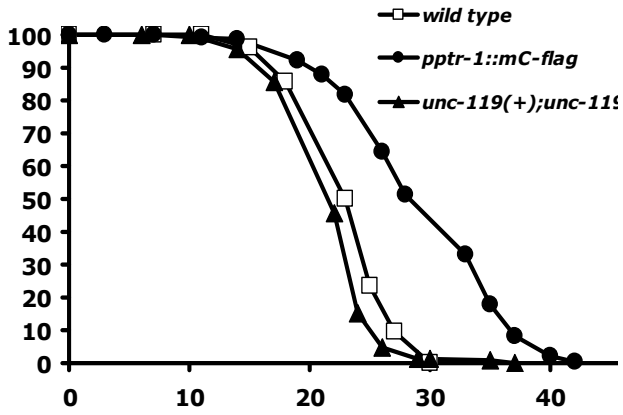
As a corollary to this experiment, we next looked at the effect of *pptr-1* RNAi on DAF-16 nuclear localization. For this, we generated a strain with a *daf-2(e1370*);*daf-16::gfp* strain. At the permissive temperature of 15^{o}C, DAF-16::GFP is excluded from the nucleus in the *daf-2(e1370);daf-16::gfp* strain. However, at the non-permissive temperature of 25 ^{o}C, progressive nuclear localization of DAF-16::GFP is observed. We grew *daf-2(e1370);daf-16::gfp* worms on either vector, *pptr-1* or *daf-18* RNAi and measured the extent of nuclear localization at 25 ^{o}C. We find that *pptr*-1 RNAi significantly reduced DAF-16 nuclear localization, similar to the effect of *daf-18* RNAi (Table 8B). Together, these experiments suggest that changes in PPTR-1 levels affect the activity of AKT-1 and as a result, modulate DAF-16 sub-cellular localization

DAF-16 target genes

DAF-16 regulates the transcription of many downstream genes such as *sod-3*, *hsp-12.6*, *sip-1* and *mtl-1* (Furuyama, Nakazawa et al. 2000; Lee, Kennedy et al. 2003; McElwee, Bubb et al. 2003; Murphy, McCarroll et al. 2003; Oh, Mukhopadhyay et al. 2006). We next tested the effects of *pptr-1* RNAi on these DAF-16 transcriptional targets. We first tested *sod-3* which has been shown to be a direct target of DAF-16

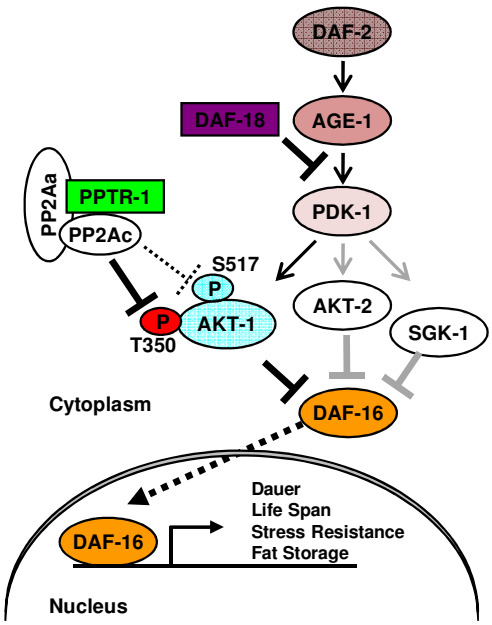
by chromatin immunoprecipitation (Oh, Mukhopadhyay et al. 2006) and its expression changes in response to modulation of the IIS pathway (Furuyama, Nakazawa et al. 2000; Libina, Berman et al. 2003). We grew a *daf-2(e1370);Psod-3::gfp(muIs84)* strain on either vector, *daf-18* or *pptr-1* RNAi to look at the effect on GFP expression. Similar to worms grown on *daf-18* RNAi, *pptr-1* RNAi reduces expression of GFP (Table 9A). Therefore, modulation in the levels of *pptr-1* can affect the expression of direct DAF-16 target genes.

We further analyzed the expression of known DAF-16 target genes by quantitative RT-PCR in a *daf-2(e1370)* mutant background. As a control, we analyzed whether each of these target genes expressed in a *daf-16*-dependent manner as previously reported (McElwee, Bubb et al. 2003; Murphy, McCarroll et al. 2003; Oh, Mukhopadhyay et al. 2006). As shown in Table 9B, *daf-16* RNAi dramatically suppressed the expression levels of these genes. Next, we tested the effects of either *pptr-1* or *daf-18* RNAi on the expression of these genes. We found that *pptr-1* RNAi also suppressed the expression of these genes to a level similar to *daf-18* RNAi. Taken together, our data suggests that PPTR-1 positively regulates DAF-16 nuclear localization and thereby its activity.



Overexpression of PPTR-1 significantly increases the life span of wild type worms. Mean life span of wild type worms is 23.9 ± 0.3 days (n=154), *pptr-1::mC-flag* is 30.1 ± 0.5 days (n=202), p<.0001, and the *unc-119(+); unc-119(ed3)* control strain is 22.6 ± 0.3 days (n=145).

Figure 28



Model illustrating the role of PPTR-1 in the insulin/IGF-1 signaling pathway. Signals from DAF-2 transduced by a PI3-kinase pathway then leads to the phosphorylation and activation of downstream serine/threonine kinases such as PDK-1, AKT-1, AKT-2 and SGK-1.

PPTR-1, the PP2A holoenzyme regulatory subunit, regulates the dephosphorylation and activation status of AKT-1 at T350 when upstream signaling is reduced. This in turn affects the nuclear translocation, activity of DAF-16 and results in the expression of genes involved in life span, dauer formation, stress resistance and fat storage

Figure 29

Discussion

The insulin/IGF-1 (IIS) signaling pathway regulates growth, metabolism and longevity across phylogeny. Given the large number of cellular processes that this pathway controls, understanding the mechanisms that modulate IIS is of paramount importance. IIS is a well-studied kinase cascade but few phosphatases in the pathway are known. Identification of these phosphatases, especially those that counterbalance the activity of the kinases, will provide a better insight into the regulation of this important pathway. *C. elegans* is an excellent system amenable to genetic manipulations including RNAi. In addition, the worm IIS pathway controls several well-defined phenotypes such as life span and dauer formation that can be easily quantitated. Therefore, to identify novel phosphatases regulating the IIS pathway, we performed a directed RNAi screen using dauer formation as an output. We specifically looked for serine/threonine phosphatases, as the majority of phosphorylations in the cell, including the insulin signaling pathway, occur on serine or threonine residues (Moorhead, Trinkle-Mulcahy et al. 2007).

We identified *pptr-1* as a top candidate in our screen. This gene encodes a protein that bears homology to the mammalian B56 family of PP2A regulatory subunits (Janssens, Longin et al. 2008). PP2A itself is a ubiquitously expressed phosphatase that is involved in multiple cellular processes including the regulation of insulin signaling by direct dephosphorylation of Akt (Andjelkovic, Jakubowicz et al. 1996; Resjo, Goransson et al. 2002; Ugi, Imamura et al. 2004). Specificity of PP2A to its various cellular targets is achieved by its association with distinct regulatory subunits. Our studies provide a mechanistic insight into how the *C. elegans* PP2A regulatory subunit PPTR-1 modulates insulin signaling by specifically regulating AKT-1 phosphorylation and activity in the context of a whole organism. Furthermore, we show that this mechanism of regulation is conserved in mammals.

We identify PPTR-1 as a novel and integral component of the *C. elegans* IIS pathway. In our model (Figure 29), PPTR-1 acts to negatively regulate signals transduced through the IIS pathway, ultimately

controlling the activity of the FOXO transcription factor DAF-16. Under low signaling conditions, DAF-16 is able to translocate to the nucleus and transactivate or repress its downstream targets. It is well established that AKT modulates DAF-16 sub-cellular localization. Thus, the activity of AKT-1, as governed by its phosphorylation status, directly translates into the activity of DAF-16. In this study, we show that PPTR-1 directly interacts with AKT-1 and regulates its activity by modulating its phosphorylation, predominantly at the Thr 350 site. Less active AKT-1 results in increased DAF-16 nuclear localization. Indeed, DAF-16 is found to be more nuclear throughout the worm when PPTR-1 is overexpressed. As a corollary, knocking down *pptr-1* by RNAi results in less nuclear DAF-16 as well as reduced expression of DAF-16 target genes such as *sod-3*, *hsp-12.6, mtl-1* and *sip-1*. These genes are known to play a combinatorial role in adaptation to various stresses, leading to enhanced dauer formation and increased life span. Consistent with the decreased levels of these important genes, *pptr-1* RNAi results in a significant decrease in the dauer formation, life span as well as thermotolerance of *daf-2*(*e1370)* worms. In addition, *pptr-1* also regulates other DAF-16-dependent outputs of the IIS pathway such as fat storage. Thus, we find that normal levels of *pptr-1* are important under low insulin signaling conditions. However, *pptr-1* RNAi does not affect IIS pathway-associated phenotypes in wild type worms. There could be several reasons for this observation. Firstly, under normal signaling conditions, AKT-1, AKT-2 as well as SGK-1 are active and negatively regulate DAF-16. Therefore, changes in the AKT-1 activity alone brought about by *pptr-1* RNAi may not have a significant effect on DAF-16-dependent phenotypes. Secondly, PPTR-1 itself may be negatively regulated by the IIS pathway, leading to increased AKT-1 phosphorylation. Along similar lines, in mammals, insulin signaling can downregulate the expression and activity of the PP2A catalytic subunit (Srinivasan and Begum 1994; Hojlund, Poulsen et al. 2002; Ugi, Imamura et al. 2004). Thus, under normal conditions, further down regulation of *pptr-1* by RNAi may have no effect. We speculate that in *C elegans*, in response to changing environmental cues, PPTR-1 helps to downregulate the insulin signaling pathway to promote DAF-16 activity, enabling the worm to either enter diapause or enhance its tolerance to stress as adults.

In mammals, Akt controls a myriad of secondary signaling cascades that regulate glucose transport, protein synthesis, genomic stability, cell survival and gene expression (Toker and Yoeli-Lerner 2006). Previous studies have implicated roles for PP2A and PHLPP phosphatases in the negative regulation of Akt (Kuo, Huang et al. 2008). The PP2A inhibitor Okadaic acid can increase Akt phosphorylation predominantly at Thr 308 and enhance glucose transport in adipocytes (Rondinone, Carvalho et al. 1999). Consistent with this, our results show that siRNA knockdown of the PP2A catalytic subunit and more importantly, the B56β regulatory subunit results in enhanced Akt phosphorylation at Thr 308 in 3T3-L1 adipocytes. Thus, our study points at the remarkable functional conservation of the B56/PPTR-1 regulatory subunit of PP2A in regulating AKT phosphorylation between *C. elegans* and higher mammals. In worms, we also see a modest effect on Ser 517 (equivalent to mammalian Ser 473) phosphorylation by PPTR-1 overexpression. However, we do not observe a difference in Ser 473 phosphorylation in adipocytes. This difference may be explained by the fact that in worms, we are determining the phosphorylation of AKT-1 in the context of a whole organism. Additionally, it has been shown in mammals that the pdk dependent phosphorylation (at T308) is necessary and also influences the stability of the carboxy terminal phosphorylation at S473. Conversly, Okadaic acid (a PP2A inhibitor) increases the phosphorylation of the T308 site predominantly with a modest effect on the phosphorylation status of S473 site. These findings are consistent with our observation in worms that overexpressing PPTR-1 dramatically decreases the phosphorylation status of T350 (homologous to T308 in mammals) while only modestly affecting the S517 (homologous to S473). (Toker and Newton 2000; Gao, Furnari et al. 2005). We do not see a role for the PP2A B55 subunit (*sur-6)* in the *C. elegans* IIS pathway. However, a recent report using cell culture has implicated the mammalian B55 in the regulation of AKT (Kuo, Huang et al. 2008).

In mammals, Akt regulates a myriad of secondary signaling cascades that regulate glucose transport, protein synthesis, cell division, genomic stability, glycogen synthesis, cell migration, cell survival and gene expression {Toker, 2006 #16933}. Previous studies have implicated roles for PP2A and PHLPP phosphatases

in the negative regulation of Akt {Kuo, 2008 #15118}. The B56 family of PP2A regulatory subunits is known to regulate several pathways such as Wnt signaling, apoptosis and Erk signaling {Eichhorn, 2008 #14064}. In this study, we show that the B56/PPTR-1 regulatory subunit of PP2A specifically modulates Akt phosphorylation and hence its activity, thus showing remarkable functional conservation between *C. elegans* and higher mammals. Although we show here that *pptr-1* is an integral component of *C. elegans* IIS and lifespan regulation it remains to be seen if such a function is also conserved in mammals. However our findings could probably have significant implications in human diseases.

Targeting specific B56 isoforms to Akt could potentially be used to downregulate the phosphorylation/activity of AKT even when upstream signaling is present. For example, PTEN has been shown to be mutated in a large number of cancers almost as frequently as p53 itself. The major downstream consequence of PTEN mutations is hyper-phosphorylated AKT, reducing AKT activity in this context could have an important therapeutic utility. (Smith U et al, Ann NY Acad Sci, 1999), Sasaoka et al, Pharmacol Ther, 2006, Rondinone et al, Diabetologica, 1999).

Dysregulation of Insulin signaling is also a feature in Diabetes. A major cause of type-II (insulin resistant) diabetes has been shown to be desensitization of signaling downstream of IRS. Studies with human adipocytes from type II diabetics has shown that Okadaic acid (a PP2A inhibitor), can rescue the insulin sensitivity of these adipocytes. Okadaic acid restores insulin sensitivity by its ability to act downstream of the IRS proteins directly on Akt1, by inhibiting PP2A activity and indirectly increasing Akt1 phosphorylation and glucose transport (Rondinone et al, Diabetologica, 1999). Small molecule inhibitors of specific B56 isoforms could be potential new therapeutic targets for type-II diabetes.

CHAPTER IV

Materials and Methods

Strains

All strains were maintained at 15°C using standard *C. elegans* techniques (Stiernagle 2006). Double mutants were made using standard genetic methods as described below. For all RNAi assays, the worms were grown for at least two full generations on the RNAi bacteria.

For making double mutants, *daf-2(e1370)* males were mated to either *akt-1(ok525), akt-2(ok393)* and *sgk-1(ok538)* hermaphrodites, respectively. A total of 40 F1 progeny were singled onto individual plates and allowed to have progeny at 25 °C. From the F2 progeny on each plate, dauers were selected and allowed to recover at 15 °C. The adult worms were singled and transferred to 25 °C and allowed to have progeny. The F3 progeny formed 100% dauers at 25°C, and parents were tested for *akt-1(ok525), akt-2(ok393)* or *sgk-1(ok538)* deletion by PCR. The primers used for the PCR analysis are listed in Table 11.

For making the *daf-2(e1370)*;*daf-3(mgDf90)* double mutant, *daf-2(e1370)* males were crossed to *daf-3(mgDf90)* hermaphrodites. The F1 progeny males were selected and mated back to *daf-3(mgDf90)* hermaphrodites. Forty F2 progeny were transferred to individual plates and incubated at 25 °C. After 4-5 days, parents were selected from plates where the F3 progeny were 100% dauers and the *daf-3* deficiency was checked by PCR, as described previously (Patterson, Koweek et al. 1997). Dauers were recovered to establish the strain.

For co-localization and immunoprecipitation experiments, *pptr-1*::*mC-flag* males were mated to *myo-3::gfp* (Fire, Xu et al. 1998), *akt-1::gfp* (Sp209) (Paradis and Ruvkun 1998), *akt-2*::*gfp*;*unc-119(+);unc-119(ed3), sgk-1*::*gfp* (BR2773; kind gift from Ralf Baumeister) (Hertweck, Gobel et al. 2004) and *daf-16::gfp*

(kind gift from Ruvkun Lab) hermaphrodites, respectively. F1 progeny were selected and subsequently F2 worms homozygous for both GFP and *mCherry* were selected under a fluorescence microscope. The extrachromosomal lines *akt-1::gfp* (Sp209) and *sgk-1*::*gfp* (BR2777) were integrated into the genome by UV irradiation prior to making genetic doubles.

The *daf-2(e1370);daf-16::gfp* and *daf-2(e1370);Psod-3*::*gfp*(*muIs84*) strain was made by crossing *daf-2(e1370)* males to either *daf-16::gfp* (kind gift from Ruvkun Lab) or *Psod-3*::*gfp*(*muIs84*) hermaphrodites. About 40 F1 animals were transferred to individual plates and allowed to have progeny at 25°C. From the progeny, F2 dauers were selected from each plate and allowed to recover at 15°C. The recovered adult worms were then checked for the presence of GFP, and GFP-positive worms were transferred to individual plates and incubated at 25 °C. Plates where 100% of the progeny were dauers and GFP positive were selected and established as the strain for the assays.

Strain	Strain Number	Comment
daf-2(e1370);akt-1(ok525)	HT1547	
daf-2(e1370);akt-2(ok393)	HT1548	
daf-2(e1370);sgk-1(ok538)	HT1648	
daf-2(e1370);daf-3(mgDf90)	HT1607	**Crossed by Sridevi**
akt-1::gfp	HT1632	Integrated *SP209 Pakt-1::akt-1::gfp*, *rol-6* from Ruvkun Lab (Paradis and Ruvkun, 1998)
akt-2::gfp; unc-119(+); unc-119 (ed3)	HT1604	Integrated Line
sgk-1::gfp	HT1634	Integrated BR2777 *Ex[sgk-1::gfp]* from Baumeister Lab (Hertweck et al., 2004)
daf-2(e1370);daf-16::gfp	HT1531	Crossed P*daf-16a::daf-16::gfp* from Ruvkun Lab to *daf-2(e1370*) (Lee et al., 2001)
pptr-1::mC-flag	HT1630	Integrated Line
akt-1::gfp; pptr-1::mc-flag	HT1644	
akt-2::gfp; pptr-1::mc-flag	HT1645	
sgk-1::gfp; pptr-1::mc-flag	HT1635	
daf-16::gfp; pptr-1::mc-flag	HT1646	
myo-3::gfp; pptr-1::mc-flag	HT1647	Crossed PD4251 to *pptr-1 ::mc-flag* (Fire et al., 1998)
daf-2(e1370); pptr-1::mc-flag	HT1641	
daf-2(e1370) ; Psod-3 ::gfp	HT1643	Crossed *muIs84*[pAD76(*sod-3::GFP*)] to *daf-2(e1370)* (Libina et al., 2003)
unc-119(+) ; unc-119(ed3)	HT1638	Extrachromosomal Array Line (**made by Eunsoo**)
daf-2(e1370) ; unc-119(+) ; unc-119(ed3)	HT1642	Extrachromosomal Array Line (**made by Eunsoo**)

Table 10

Preparation of RNAi plates

RNAi plates were prepared by supplementing Nematode Growth Media (NGM) media with 100 µg/ml ampicillin and 1 mM IPTG. After pouring, the plates were kept at room temperature (RT) for 5 days to dry. RNAi bacteria were grown overnight at 37°C in LB media supplemented with 100 µg/ml ampicillin and 12.5 µg/ml tetracycline. The next day, the cultures were diluted (1:50) in LB containing 100 µg/ml ampicillin and grown at 37°C until an OD_{600} of 0.9. The bacterial pellets were resuspended in 1X PBS (phosphate-buffered saline) (for RNAi screen) or PBS with containing 1 mM IPTG for further experiments. About 200 µl of the bacterial suspension was seeded onto the RNAi plates. The seeded plates were dried at RT for 3 days and stored at 4°C.

C. elegans assays

C. elegans assays were modified from previously published methods (Kimura, Tissenbaum et al. 1997; Henderson and Johnson 2001; Libina, Berman et al. 2003; Oh, Mukhopadhyay et al. 2005; Oh, Mukhopadhyay et al. 2006). For detailed description of dauer, life span, fat storage, heat-stress, DAF-16:GFP translocation and Psod-3::GFP expression assays see below:

Dauer assays

For the dauer assays, approximately 5 L4 or young adult worms were transferred to the RNAi bacteria and maintained at 15 ^{o}C. F2 adult worms were then picked to a fresh RNAi plate and allowed to lay eggs. About 120 eggs were picked from these plates onto 2 or 3 fresh plates containing the RNAi bacteria and incubated at the indicated temperatures. The plates were scored for the presence of dauers or non-dauers after 3.5-4 days, unless indicated otherwise. For assays involving *daf-2(e1370);sgk-1(ok538)*, the strain is slow growing with a prolonged L1/L2 arrest and only forms dauers on vector RNAi after 7-8 days. Similarly, *daf-2(e1370);akt-1(ok525)* worms grown on vector and *pptr-1* RNAi were also scored after 7-8 days. The *pdk-1(sa680)* worms have an Egl phenotype. For the *pdk-1(sa680)* dauer assays, eggs were

obtained by hypochlorite treatment (Stiernagle 2006) of gravid adults worms grown on vector, *daf*-18 and *pptr*-1 RNAi plates grown at 15^{o}C.

Life span assays

All life span analyses were performed at 15ºC unless indicated otherwise. For RNAi lifespan worms were grown for 2 generations on the RNAi plates, synchronized by picking eggs on to fresh RNAi plates and allowed to grow for several days until they became young adults. Approximately 60 young adult worms were transferred to each of 3 RNAi plates for every RNAi clone tested. For life span experiments with overexpression strains, approximately 60 young adult worms were transferred to 3 fresh OP50 plates for every strain tested. Life spans were performed on RNAi plates or OP50 plates overlaid with 5-fluorodeoxyuridine (FUDR) to a final concentration of 0.1 mg/ml of agar(Hosono, Mitsui et al. 1982). We observed significantly fewer worms bursting at 15ºC by transferring young adult animals rather than L4 animals to FUDR plates. Worms were then scored as dead or alive by tapping them with a platinum wire every 2-3 days. Worms that died from vulval bursting were censored. Statistical analyses for survival were conducted using the standard chi-squared-based log rank test.

Heat stress assays

Wild type and *daf-2(e1370)* animals were maintained on RNAi bacteria at 15ºC. From these plates, approximately 30 young adult worms were picked onto the respective RNAi plates. They were shifted to 20^{o}C for 6 hrs. The plates were then transferred to 37^{o}C and heat stress-induced mortality was determined every few hours till all the animals were dead. Statistical analyses for survival were conducted using the standard chi-squared-based log rank test.

Fat staining

Sudan black staining of stored fat was performed as previously described (Kimura, Tissenbaum et al. 1997). Briefly, wild type and *daf-2(e1370)* worms on RNAi plates were synchronized by picking eggs on

to fresh RNAi plates and grown until the L3 stage. The worms were then washed off the plates and incubated in M9 buffer for 30 minutes on a shaker at RT. After 3 washes with M9 buffer, the worms were fixed in 1% paraformaldehyde. The worms were then sequentially dehydrated by washes in 25%, 50% and 70% ethanol. Saturated Sudan Black (Sigma, USA) solution was prepared fresh in 70% ethanol. The fixed worms were incubated overnight in 250uL of Sudan Black solution, on a shaker at RT, mounted on slides and visualized using the Zeiss Axioscope 2+ microscope.

DAF-16::GFP localization assay

The *daf-2(e1370);daf-16::gfp* strain was maintained at 15 ^{o}C on vector, *daf-18* or *pptr-1* RNAi plates. About 20-25 L4 or young adults were transferred to fresh RNAi bacteria and the plates were shifted to 25 ^{o}C for 1hr. The worms were then visualized using Zeiss Axioscope 2+ microscope. Worms were classified into four categories based on the extent of DAF-16::GFP nuclear-cytoplasmic distribution.

+: completely cytoplasmic;

++: nuclear in some tissues but cytoplasmic in majority of the tissues;

+++: cytoplasmic in some tissues but nuclear in majority of the tissues;

++++: nuclear localization in all tissues (Hertweck, Gobel et al. 2004)

Psod-3::GFP expression

daf-2(e1370);Psod-3::*gfp*(*muIs84*) worms were grown at 15^{o}C on RNAi plates as described above. About 25-30 L4/young adults were transferred to fresh RNAi plates and shifted to 25^{o}C for 2 hrs. The expression of GFP was visualized using Zeiss Axioscope 2+ microscope. Worms were classified into three categories based on the intensity of GFP expression.

High: bright GFP expression seen throughout the worm;

Medium: Medium GFP expression in the worm body;

Low: weak or barely detectable GFP expression in the body.

GFP expression in the head region does not change dramatically.

Construction of *pSCFTdest*

The *pSCFTdest* vector was derived from *pPD95.75* vector (Fire lab). Briefly, the *mcherry* gene was amplified from the *mCherry* vector (McNally, Audhya et al. 2006) using primers listed in Table 11. The reverse primer added a TEV site with a spacer and a minimal flag tag (Johnson, Liu et al. 2002)to the C-terminus of *mCherry*. The amplified product was restriction digested with *Kpn*I and *EcoR*I and ligated to *pPD95.75* at *Kpn*I/*EcoR*I (replacing the *gfp* gene in the vector) to give *pSCFT* (CFT-Colocalization Flag tag) plasmid. The R4-R2 gateway cassette from *pdestMB14* (Reboul, Vaglio et al. 2003) was PCR-amplified using *ccdb* primers (Table 11). The amplicon was restriction digested with *Kpn*I, producing an insert with one blunt end and another *Kpn*I-compatible end. *pSCFT* was then digested with *Sma*I and *Kpn*I and the blunt end/KpnI insert was ligated into the cut vector to make pSCFTdest.

Transgenic worms

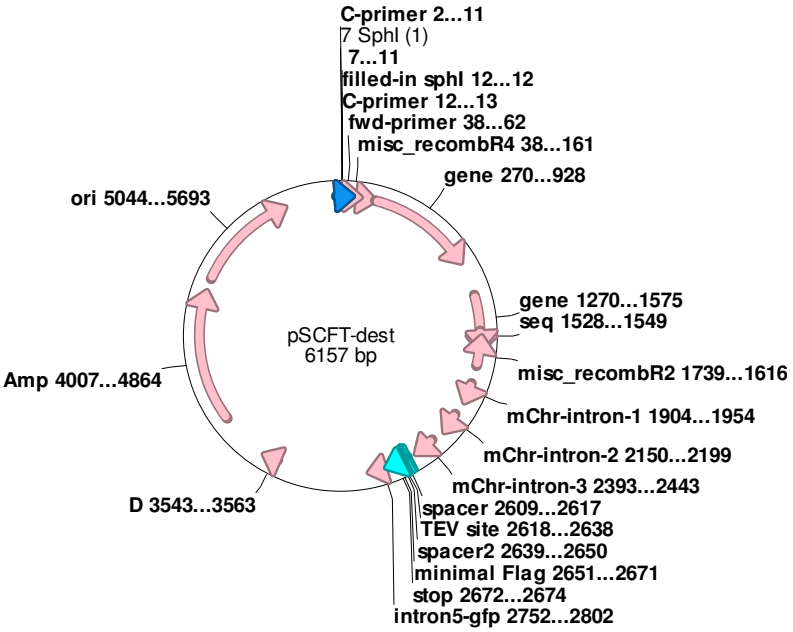
A 3 kb sequence of the *pptr-1* promoter and the *pptr-1* ORF were cloned into separate entry vectors (Walhout, Temple et al. 2000; Reboul, Vaglio et al. 2003) using Gateway Technology (Invitrogen, USA) and confirmed by DNA sequencing. The promoter and ORF were then combined using multi-site Gateway cloning into the *pSCFTdest* vector (Supplemental Methods) to create the *pSCFT-pptr-1*. An *unc-119* promoter::ORF fusion mini-gene was constructed as described earlier (Maduro and Pilgrim 1995) and cloned into *pUC-19* vector between *EcoR*I sites. The *unc-119* mini-gene insert was then excised using *EcoR*I restriction digestion, gel-purified, blunt ended with T4 DNA Polymerase (Roche Biochemicals, USA) and cloned into *pSCFT-pptr-1* (at the filled-in *Sph*I site) giving rise to the *pSCFT-pptr-1-unc-119* vector. This construct was used in biolistic transformation (Biorad, USA) of *unc-119(ed3)* mutants (Maduro and Pilgrim

1995; Praitis 2006). PPTR-1 over-expression integrated lines were back-crossed four times to wild-type and used for further analysis.

For the *akt-2::gfp* construct, a 3 kb sequence of the akt-2 promoter was cloned into the corresponding entry vector and the *akt-2* ORF from the ORFeome were combined using multi-site Gateway technology into the R4-R2 destination vector (Reboul, Vaglio et al. 2003) to create *akt-2::gfp- unc-119(+)* vector. This vector was verified by restriction digestion and integrated transgenic lines were obtained by micro-particle bombardment (Maduro and Pilgrim 1995; Praitis 2006).

A

pSCFT Vector map



B

pSCFT-pptr-1-unc-119 vector map

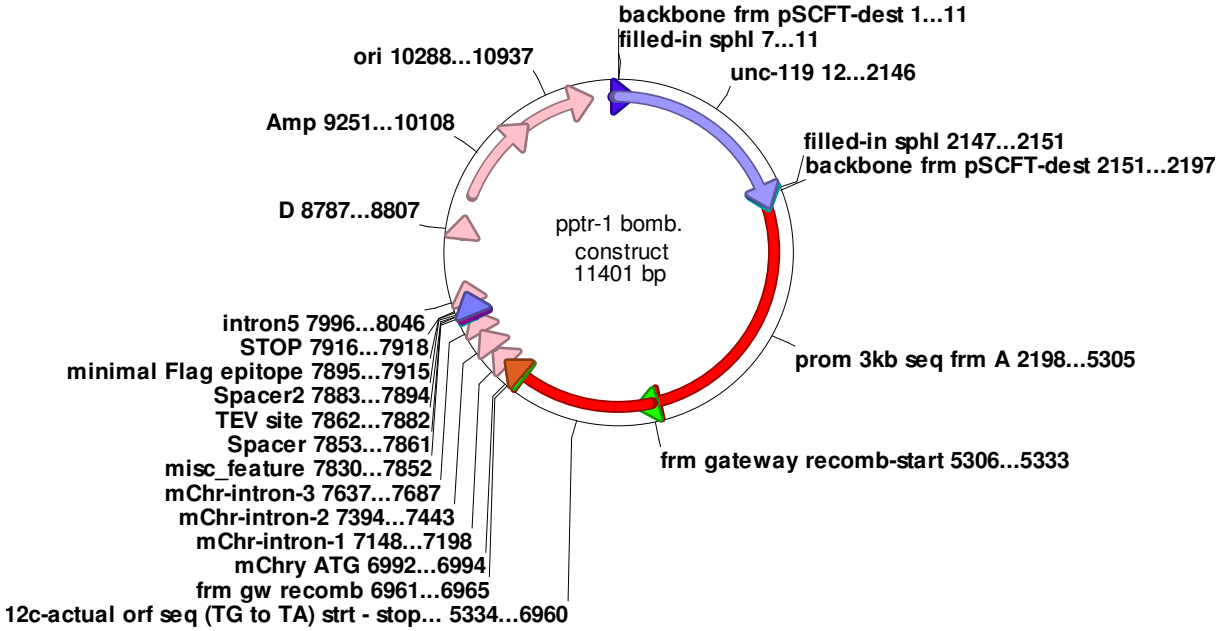


Figure 30

C.elegans AKT-1 phospho-specific antibodies:

Phospho antibodies were prepared and affinity purified by 21st Century Biochemicals (Marlboro, MA). Briefly, rabbits were immunized with phospho-peptides (mixture of AKT pT350 PP1 and AKT pT350 PP2 for T350 and pS517PP1 for S517) and after 6 boosts, the rabbit serum was immunodepleted with a peptide column conjugated to non-phosphorylated peptide (AKT T350 NP or AKT S517 NP). Following immunodepletion, the sera was affinity purified with Phospho-peptide AKT pT350 PP1 (for T350 site) or AKT pS517 PP1 (for S517 site).

The peptides used were:

AKT pT350 PP1: Ac-TS[pT]FCGTPEYK-amide(injected)

AKT pT350 PP2: CSYGDKTS[pT]FSGTPEY-amide(injected)

AKT T350 NP: CSYGDKTSTF[C/S]GTPEY-amide-used for (immunodepletion)

AKT pS517 PP1: Ac-CSNFTQF[pS]FHNVMGS-amide-conjugated (injected)

AKT S517 NP: Ac-CSNFTQFSFHNVMGS-amide-conjugated (immunodepletion)

C. elegans immunoprecipitation (IP) and western blotting:

Transgenic worms were grown in three 100 mm plates seeded with OP50 bacteria at 20 ^{o}C. Worms were harvested by washing with M9 buffer and pellet collected by centrifugation. The pellet was resuspended in 250 µl lysis buffer (20 mM Tris-Cl, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 25 mM β-glycerophosphate, Protease inhibitor cocktail (Roche Biochemicals, USA), pH 7.4). The worms were sonicated with Bioruptor (Diagenode, USA) using maximum power output (1 min sonication, 2 min off-repeated 10 times). The lysate was cleared by centrifugation and protein content estimated by Bradford method. Lysate equivalent to 1.5 mg total protein was pre-cleared with 50 µl of protein-G agarose beads, fast flow (Upstate, USA) and then immunoprecipitated overnight at 4 ^{o}C using either anti-GFP monoclonal

antibody (Sigma, USA) or anti-FLAG M2 gel (Sigma, USA). The following morning, 50 µl protein-G agarose beads, fast flow were added to the GFP IP to capture the immune complex. The agarose beads were then washed 5 times with lysis buffer. Following this step, the beads were boiled in Laemelli's buffer.

For western blot analysis, immunoprecipitated protein samples was resolved on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in TBST (Tris Buffered Saline containing 0.05% Tween 20, ph 7.4) containing 5% non-fat milk at RT for 1 hour. Membranes were then washed with TBST and incubated overnight with 1:1000 dilutions of antibodies in TBST containing 5% non-fat milk 4 ºC. Membranes were washed 3 times with TBST and then incubated with TBST containing 5% non-fat milk containing a 1:10,000 dilution of the secondary antibody. Antibodies used for western were:

Living Color DsRed antibody (Clontech, USA; Catalog no. 632496)

Living Color Rabbit polyclonal GFP antibody (BD Biosciences, USA; Catalog no. 632460)

Monoclonal mAb 3e6 GFP antibody (Invitrogen, USA; Catalog no. A11120)

Anti-FLAG M2 Affinity Gel (Sigma, USA; Catalog no. A2220).

C. elegans phospho-AKT western blotting:

Transgenic worms were grown at 20 ^{o}C in 3-4 large (100 mm) plates seeded with OP50. Worms were collected by washing with 1 X PBS and the pellet was then immediately frozen in dry ice. Around 500 µl lysis buffer, supplemented by Sigma Phosphatase inhibitor cocktails I and II (50x) and Protease inhibitor cocktail (Roche Biochemicals, USA) was added to the pellet and sonicated using a Misonix (3000) sonicator (Misonix, USA; power output set at 4, 3 pulses of 10 secs each with 1 min interval between pulses). The lysates were clarified by centrifugation at 13000 rpm for 10 mins at 4^{o}C and the protein content estimated by Quick Bradford (Pierce). About 3.5 µg of anti-GFP monoclonal antibody (3E6, Invitrogen USA) was used for each IP from lysates containing 1.7 mg protein in a volume of 1ml. IPs were performed overnight at 4^{o}C

and antibody-protein complexes were captured using 50 µl of protein-G agarose beads, fast flow (Upstate, USA) for 2 hrs at 4^{o}C. The pellets were washed 3 times with lysis buffer supplemented by protease and phosphatase inhibitors and boiled in Laemelli's buffer. The IP samples were then resolved on a 10% SDS-PAGE, western blotted and analyzed with phospho-specific antibodies (Supplemental Materials and Methods).

Mammalian cell culture and phospho-Akt Western blotting

3T3-L1 adipocytes were cultured and differentiated as previously described (Tesz, Guilherme et al. 2007). For siRNA transfections, cells from 4 days post-induction of adipocyte differentiation were used as previously described (Tang, Guilherme et al. 2006). Cells were stimulated with increasing concentrations of insulin and isolated proteins were analyzed by Western blotting Detailed procedure is described in Supplemental Materials and Methods.

Cell culture and siRNA transfection:

3T3-L1 adipocytes were cultured and differentiated in complete Dulbecco's modified Eagle's medium (10% fetal bovine serum, 50 units/ml penicillin, and 50 g/ml streptomycin) as previously described (Tesz, Guilherme et al. 2007). For siRNA transfections, cells from 4 days post-induction of adipocyte differentiation were used as previously described (Tang, Guilherme et al. 2006). Briefly, 1.125 x 10^{6} cells were electroporated using 6 nmol of siRNA and then plated in 5 wells of a 12-well plate. Cells were recovered in complete DMEM and were cultured for 48 h after the transfection prior to the experiments.

Insulin stimulation and phospho-Akt western blotting

3T3-L1 adipocytes transfected with siRNA were serum-starved for 18 hours. Cells were stimulated with increasing concentrations of insulin for a period of 30 minutes. Following insulin stimulation, the cells were washed with ice-cold PBS and harvested on ice as described previously (Tesz, Guilherme et al. 2007). Protein samples were resolved on 8% SDS-PAGE and transferred to a nitrocellulose (NC) membrane as

mentioned above. Antibodies used were Phospho-Akt Ser 473 (Cell Signaling, USA; Catalog no. 9271), Phospho-Akt Thr 308 (Cell Signaling, USA; Catalog no. 9275), total Akt antibody (Cell Signaling, USA; Catalog no. 9272). Secondary antibody incubation was performed as above in 1% BSA. Changes in the phosphorylation of Akt pSer 473 and pThr 308 were quantified through densitometry using NIH ImageJ and normalized for loading against the non-phosphorylated total Akt levels.

RNA isolation and real-time PCR

RNA was isolated using Trizol (Invitrogen, USA). Briefly, worms grown on vector, *daf-16*, *daf-18* or *pptr-1* RNAi were washed off the plates using M9 buffer. Next, 0.3 ml of TRIzol reagent was added and vortexed vigorously. The RNA was then purified by phenol:chloroform:isoamylalcohol extraction and ethanol precipitation. The concentration and the purity of the RNA were determined by measuring the absorbance at 260/280 nm. To further determine the quality of the RNA, the quality of the ribosomal 28 S and 18 S was visually inspected on an agarose gel. cDNA was synthesized using 2 ug of RNA and the SuperScript cDNA synthesis kit (Invitrogen, USA).

DAPI Staining

pptr-1::mC-flag worms were washed off an OP50 plate with PBS and washed thrice by briefly spinning the worms at 3000rpm for 1 minute. The supernatant was collected and 500uL of 3% Formaldehyde (diluted with potassium phosphate buffer, KH2PO4) was added to the worm pellet. The samples were fixed for 15-20 mins with gentle shaking, and 500uL of PBS-Tween (0.1%) was added and gently mixed. The samples were then spun at 3000rpm for 1 minute and washed twice with PBS-Tween and the supernatant was removed. 2uL of DAPI (1mg/mL, Sigma D9542) added to 500uL of PBS and the samples were incubated in this solution for 15-20 minutes before mounting.

Table 11

Gene Amplified	Used for	Sequence (5'-3')
akt-1	Detecting akt-1 deletion	AGAGCGTCTATGATTAGTATTG
akt-1	Detecting akt-1 deletion	TACTTTCTGTGGAACTCCGGAG
akt-1	Detecting akt-1 deletion	AGTTTCTGACTGCACATTTGGC
akt-2	Detecting akt-2 deletion	AGTACACAGAGTGTGATAATGC
akt-2	Detecting akt-2 deletion	GCAAACTCCATCACAAAACAG
akt-2	Detecting akt-2 deletion	CACGTAGTATGACGCAAATTG
sgk-1	Detecting sgk-1 deletion	TCAGGCTTCAAATCTCTGGAA
sgk-1	Detecting sgk-1 deletion	AAACAGGCAAATACGGCATC
sgk-1	Detecting sgk-1 deletion	CACGTCATGGCAGAAAGAAA
daf-3	Detecting daf-3 deletion	CTGGCAGTCACTAACACACG
daf-3	Detecting daf-3 deletion	ACCCTCATGCCTACTGTCAG
mChry(fw-KpnI)	mCherry cloning	AGCTCG GGTACC G ATGGTCTCAAAGGGTGAAGAAG
mcherry(Rv-EcoRI-CF-tag):	mCherry cloning	TACGAATGAATTCTTAATCATCGTCCTTATAGTCCATTCCGC TAGCTCCACCCTGAAAATACAAATTCTCTCCAGCTCCCTTAT ACAATTCATCCATGCCAC
ccdb	Ccdb amplification	CAA CTT TGT ATA GAA AAG TTG AAC G
ccdb	Ccdb amplification	ATAATGGTACCTCAACTTTGTACAAGAAAGTTGAACGA
unc-119 (prom)	unc-119 amplifcation	TGTTTTGTGCCAAGCTTCAG
unc-119 (prom)	unc-119 amplification	AGTTCCGTGTGCTCTTGCTC
unc-119(cDNA)	unc-119 amplification	GGACGACCCCCATTAATTTT
unc-119(cDNA)	unc-119 amplification	ATCTGA AAGCTT TGAAAAATCATTTATTGGGATTTG
sod-3	RTPCR	GGAGTTCTCGCCGTCCG
sod-3	RTPCR	GTCGAATGGGAGATCTGGGAG
mtl-1	RTPCR	AGTGTGACTGCAAAAACAAGCAA
mtl-1	RTPCR	TCCACTGCATTCACATTTGTCTC
hsp-12.6	RTPCR	GGGATTGGCCACTTCAAAAG
hsp-12.6	RTPCR	CGTCGTCGAGGACATTGACA
sip-1	RTPCR	AAGAGATCGTTCACTCGCCAG
sip-1	RTPCR	AGCCAAGTCGACGTCCTTTG
act-1	RTPCR	CTCTTGCC CCATCAACCATG
act-1	RTPCR	CTTGCTTGGAGATCCACATC
PP2Acα	RTPCR	ATGGACGAGAAGTTGTTCACC
PP2Acα	RTPCR	CAGTGACTGGACATCGAACCT
PP2Acβ	RTPCR	ATGAGTGCCTACGGAAGTATGG
PP2Acβ	RTPCR	CAGGGCTCTTATGTGGTCCAG
B56α	RTPCR	ATTGAAGAGCCGCTTTTTAAGCA
B56α	RTPCR	TGAGGGTTTTCAGCACATTGT
B56β	RTPCR	GGGCCTACATCCGCAAACA
B56β	RTPCR	GGATCAGGACTCGAACCAGG

APPENDIX

PPTR-2, a daf-16 phosphatase?

While characterizing *pptr-1*, we found that the RNAi of the other B56 subunit – *pptr-2* also had a slight effect on dauer formation (although not reaching statistical significance) but had stronger effects on rate of growth and brood size at 15°C and 20°C. Importantly, the worms that had escaped dauer stage were far more advanced in their growth stages compared to Vector RNAi plates. These observations suggested that *pptr-2* might also have a role to play in the IIS pathway regulation like *pptr-1*. Mass RNAi screens have shown that the *pptr-1* and *pptr-2* are synthetic lethal (Tischler, Lehner et al. 2006). We observed similar lethality upon RNAi knockdown of *pptr-1* in a *pptr-2* mutant. These observations suggested that *pptr-2* acts redundantly with *pptr-1* for an essential pathway and might also have a similar effect in IIS pathway.

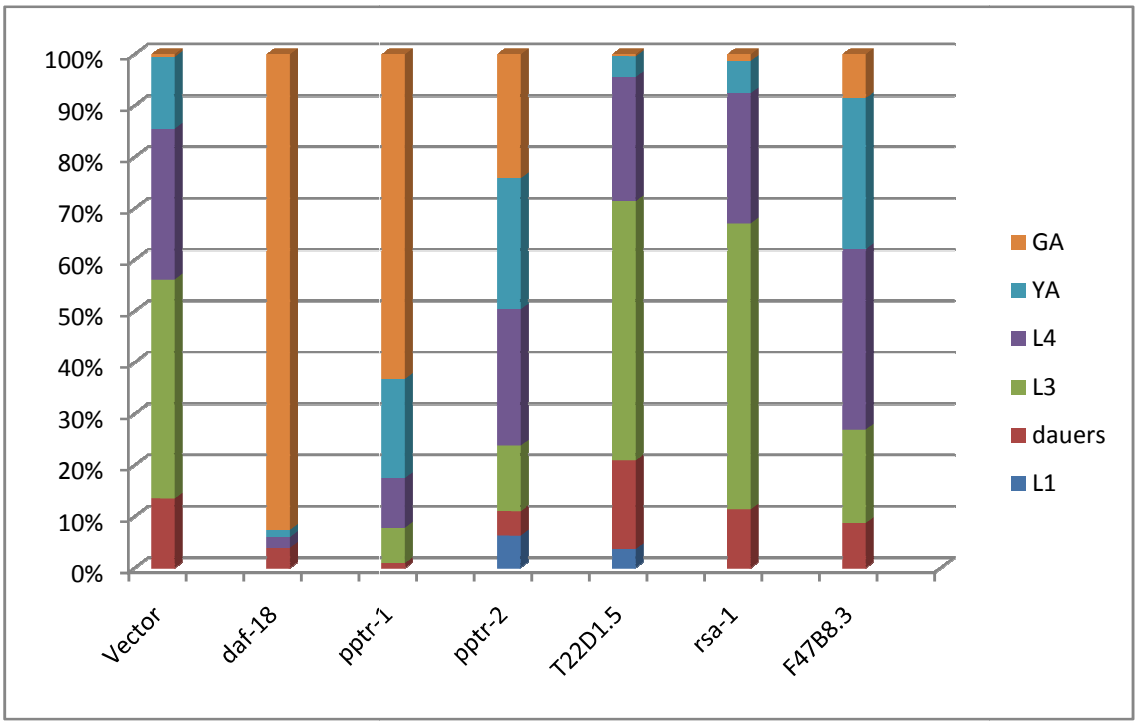
Preliminary experiments were done to determine the role of *pptr-2* in regulating IIS phenotypes in C. elegans. *pptr-2* had weaker dauer suppression effects but consistently showed mild dauer suppression and clear growth enhancement. We found that *pptr-2* RNAi suppressed *daf-2(e1370)* (Figure 1)*, daf-2;akt-2(ok393)* (Figure 2), *pdk-1(sa680)* (Figure 3 and Table I), *daf-2(e1370);akt-1(ok525)*(Figure 4, 5)*, daf-2;sgk-1(ok538)* (Figure 6) growth and dauer phenotypes to varying levels. Figure 1 shows acceleration of growth in *daf-2(e1370)* worms in *pptr-2* RNAi compared to vector. *pptr-2* RNAi cannot suppress dauer formation of *pdk-1(sa680)* at 25° C (Figure 3B, Table IB) (100% dauers in vector and *pptr-2*) and *daf-2;akt-1(ok525)* at 20 °C (Figure 4) but *pptr-2* RNAi suppressed dauer formation significantly in *pdk-1(sa680)* at 15 °C (Figure 3A) and of *daf-2(e1370);akt-1(ok525)* double at 19.2 °C (Figure 5). In contrast to *pptr-1* RNAi which was unable to suppress dauer formation of *daf-2(e1370);akt-1(ok525)* mutant even at this lower temperature(Figure 5).

We next checked if *pptr-2* RNAi has an effect on *daf-2(e1370)* lifespan. Significantly, *pptr-2* RNAi decreased *daf-2* lifespan from 33.6±0.9 in vector RNAi to 26.7±0.9 (Figure 8).

Surprisingly, *pptr-2* RNAi extended the lifespan of N2 worms significantly from 22.1±0.4 to 27.8±0.6. *pptr-1* RNAi did not affect N2 lifespan (Figure 7). We repeated the RNAi experiment in a *daf-16(mgdf50)* mutant and found that *pptr-2* RNAi did not affect the lifespan of *daf-16* mutant (Figure 9). This indicated the *pptr-2* was acting upstream or at the level of *daf-16*. The opposite effects on *daf-2* and N2 lifespan suggested an extra level of complexity in regulation of *pptr-2*. *pptr-2* and *pptr-1* belong to the B56 regulatory subunit family and they are known to be synthetically lethal in large scale RNAi screens. We therefore checked the levels of *pptr-2* in a *pptr-1* knockdown. Interestingly *pptr-2* levels went up by 3.5 fold upon *pptr-1* knockdown at 20^{o}C but not at 15^{o}C. Table 2A, 2B. It is possible that a similar regulation exists upon *pptr-2* knockdown. *pptr-2* knockdown could result in *pptr-1* upregulation in-turn leading to increased inactivation of AKT-1 and increased DAF-16 activity eventually resulting in an lifespan extension in a N2 background. But in a *daf-16* mutant background such an effect is not seen.

However, *pptr-2* knockdown also reduces *daf-2(e1370)* lifespan and can suppress dauer formation of all the mutants studied albeit at a lower efficiency. This indicates that *pptr-2* is an integral part of insulin signaling pathway and normal activity is required for *daf-2* mediated lifespan extension. The epistasis places *pptr-2* downstream of *akt-1/2* and *sgk-1* and potentially might act on *daf-16*. However, it is also possible that *pptr-2* acts on a different substrate or on multiple kinases for example *sgk-1* and *akt-2*. Interestingly, in-silico analysis of *pptr-2* interactors with the tools of Zhong et al, brings up a forkhead containing protein in *C. elegans*. More work is needed to dissect out the importance of *pptr-2* in the *C. elegans* IIS pathway.

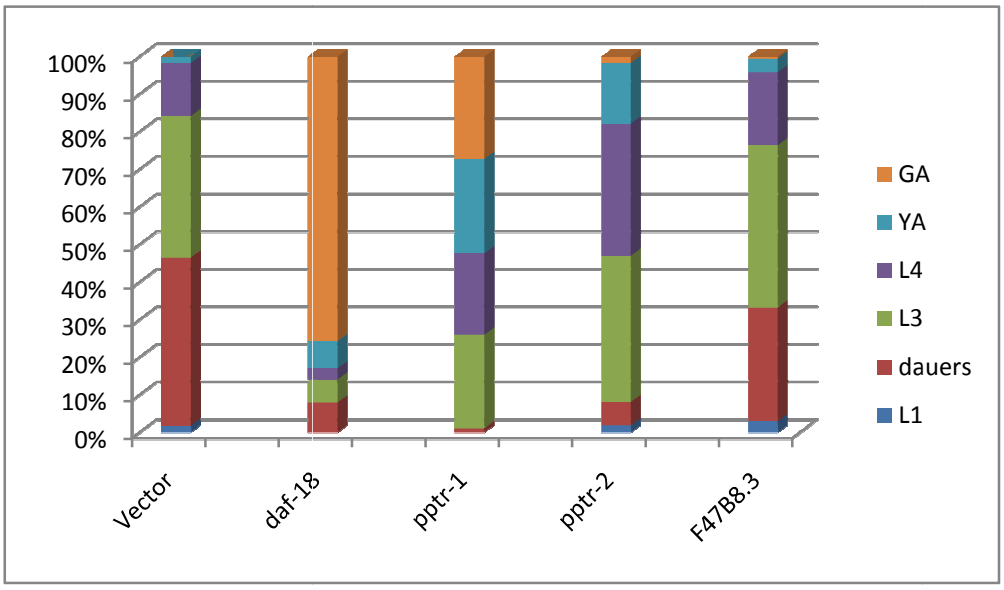
daf-2(e1370) – growth assay at 20°C



daf-2(e1370)						
	L1±Stdev	dauers±Stdev	L3±Stdev	L4±Stdev	YA±Stdev	GA±Stdev
Vector	0±0	13.5±3.4	42.7±10.4	29.4±7	14±6	0.5±0.7
daf-18	0±0	3.9±0.1	0±0	2.1±0.8	1.4±0.2	92.6±0.6
pptr-1	0±0	0.9±1.3	6.9±0.9	9.7±0.4	19.3±0.8	63.2±2.6
pptr-2	6.3±2	4.7±1.7	12.8±5.6	26.5±6.9	25.5±5.5	24.1±6.4
T22D1.5	3.8±0.8	17.9±6.6	52.4±1	25.1±5.8	4.2±0.9	0.4±0.6
rsa-1	0±0	11.4±3.1	55.8±2.4	25.4±1.5	6.3±0.7	1.3±0.1
F47B8.3	0±0	8.7±1.7	18.2±0.8	35.1±2.1	29.4±2.4	8.5±2.2

Figure 1

daf-2(e1370);akt-2(ok393) – growth assay at 20°C

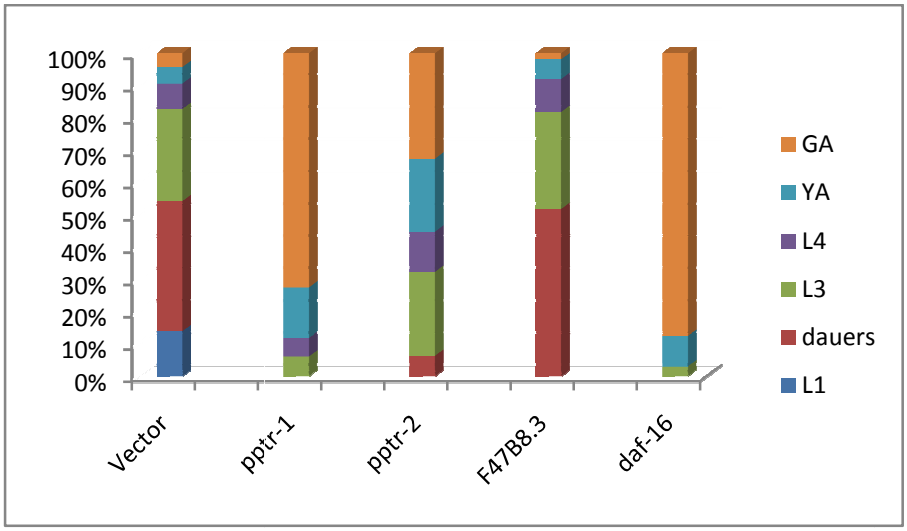


daf-2(e1370);akt-2(ok393) 20 °C						
	L1±Stdev	dauers±Stdev	L3±Stdev	L4±Stdev	YA±Stdev	GA±Stdev
Vector	1.8±0.3%	44.7±6.5%	37.6±1.4%	14.1±6.8%	1.7±1.3%	0±0%
daf-18	0±0%	8±4.1%	6.1±3.8%	3±3.1%	7.2±8.9%	75.7±19.9%
pptr-1	0±0%	1.1±0.8%	24.9±2.8%	21.6±11%	25.1±1.4%	27.2±15.9%
pptr-2	1.9±0.3%	6.2±6.3%	38.9±0.2%	35.2±5.1%	16.1±1.6%	1.7±0.1%
F47B8.3	3±0.4%	30.3±13.5%	43.2±1.2%	19.4±10.7%	3.7±3.7%	0.5±0.6%

Figure 2

A

pdk-1(sa680) 15°C



B

pdk-1(sa680) 25°C

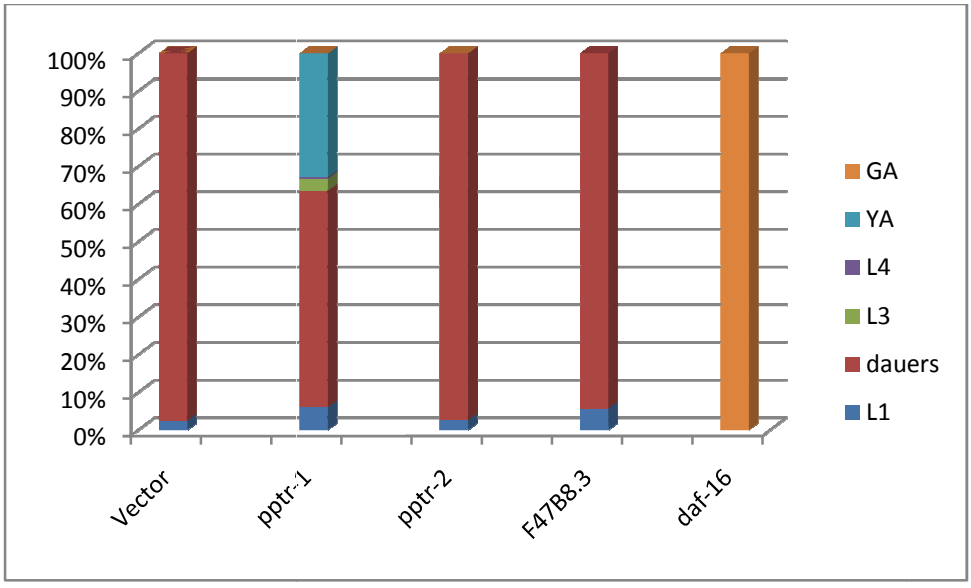


Figure 3

Table 1

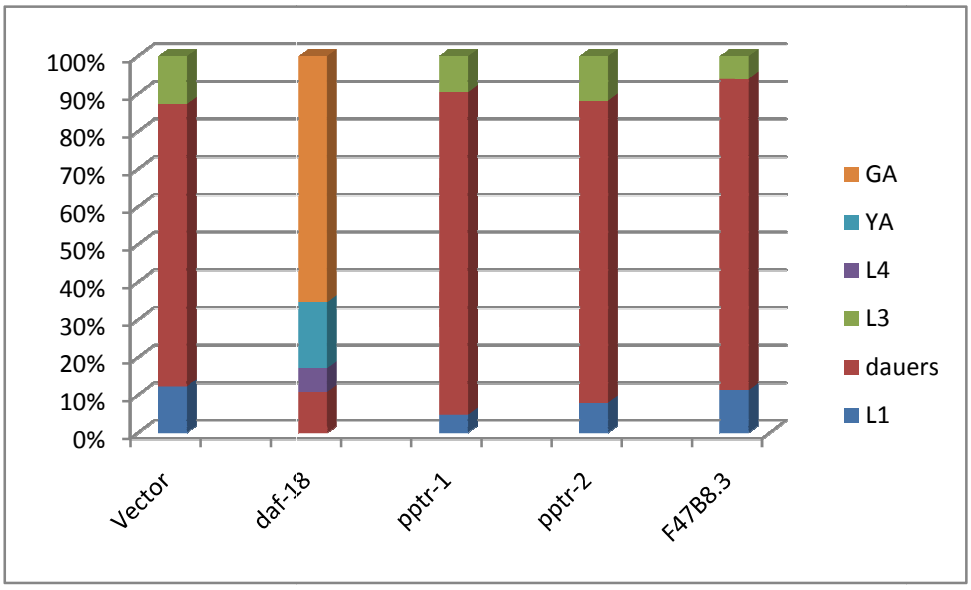
A

pdk-1(sa680) 15 °C						
pdk 15C	L1±Stdev	dauers±Stdev	L3±Stdev	L4±Stdev	YA±Stdev	GA±Stdev
Vector	13.9±9.2%	40.3±2.8%	28.5±0.4%	7.9±3.9%	5.1±1.8%	4.3±6%
pptr-1	0±0%	0±0%	6.1±3.5%	5.7±2.2%	15.7±6%	72.5±11.7%
pptr-2	0±0%	6.3±3.9%	26±2.4%	12.5±0.2%	22.6±7.2%	32.7±0.7%
F47B8.3	0±0%	51.7±0%	30±0.7%	10.2±1.1%	6.1±2.7%	1.9±0.8%
daf-16	0±0%	0±0%	3±0.5%	0±0%	9.5±5.5%	87.5±5%

B

pdk-1(sa680) 25°C						
	L1±Stdev	dauers±Stdev	L3±Stdev	L4±Stdev	YA±Stdev	GA±Stdev
Vector	2.4±3.4%	97.6±3.4%	0±0%	0±0%	0±0%	0±0%
pptr-1	6.1±0.3%	57.4±2.7%	3.2±2.2%	0.4±0.6%	32.8±0.8%	0±0%
pptr-2	2.6±0.4%	97.4±0.4%	0±0%	0±0%	0±0%	0±0%
F47B8.3	5.7±3.4%	94.3±3.4%	0±0%	0±0%	0±0%	0±0%
daf-16	0±0%	0±0%	0±0%	0±0%	0±0%	100±0%

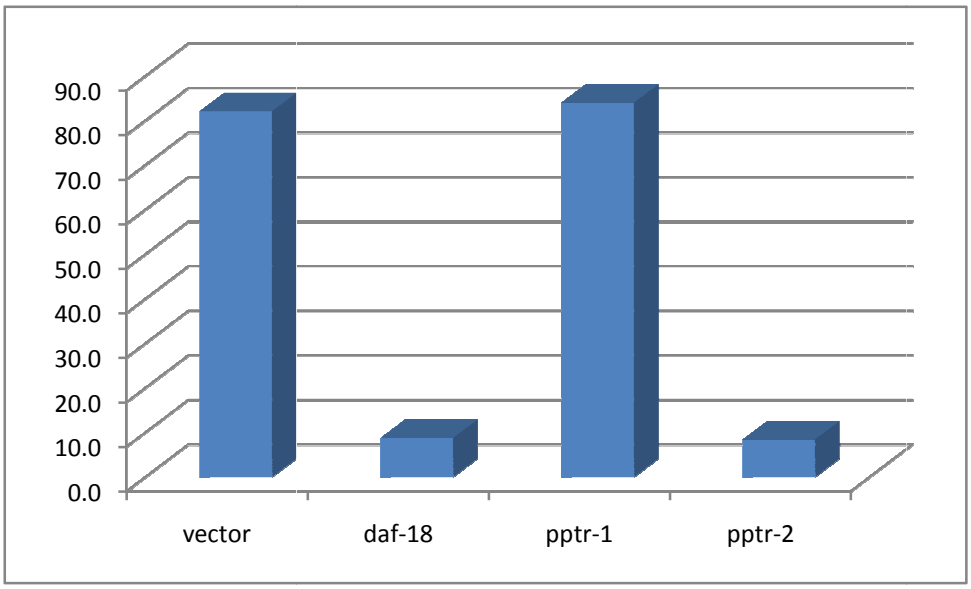
daf-2(e1370);akt-1(ok525)– growth/dauer assay at 20°C



***daf-2(e1370);akt-1(ok525)* 20 °C growth/dauer assay**						
	L1%±stdev	dauers%±stdev	L3%±stdev	L4%±stdev	YA%±stdev	GA%±stdev
Vector	5±5%	9.3±9.3%	4.3±4.3%	0	0	0
daf-18	0	1.3±1.3%	0	6.1±6.1%	6.1±6.1%	13.4±13.4%
pptr-1	3.1±3.1%	4.3±4.3%	1.2±1.2%	0	0	0
pptr-2	8.7±8.7%	9.3±9.3%	0.6±0.6%	0	0	0
F47B8.3	3.5±3.5%	0.5±0.5%	4±4%	0	0	0

Figure 4

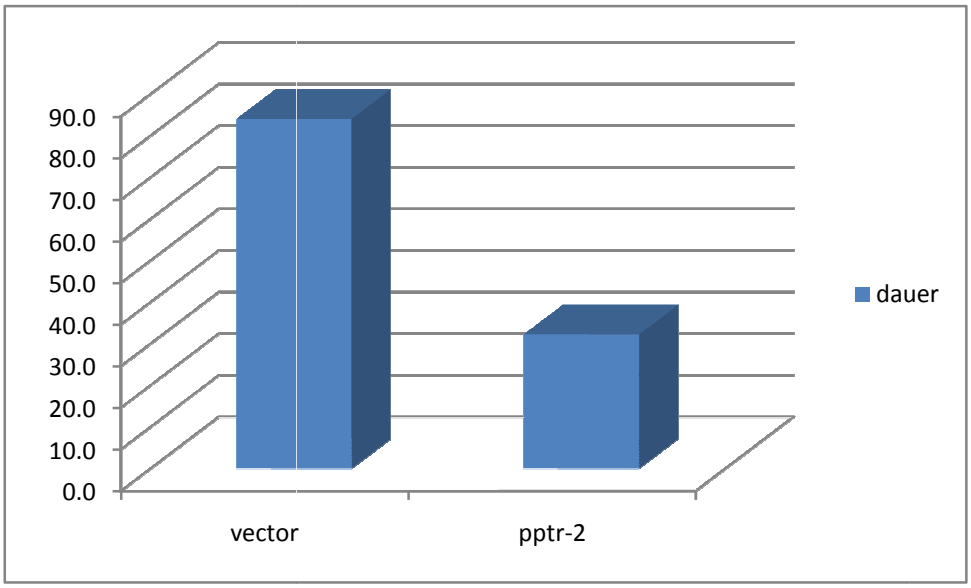
daf-2(e1370);akt-1(ok525)-dauer assay at 19.2°C



RNAi	mean	stddev	N
vector	82.1	1.0	459
daf-18	8.8	0.0	365
pptr-1	84.0	0.8	540
pptr-2	8.4	na	83

Figure 5

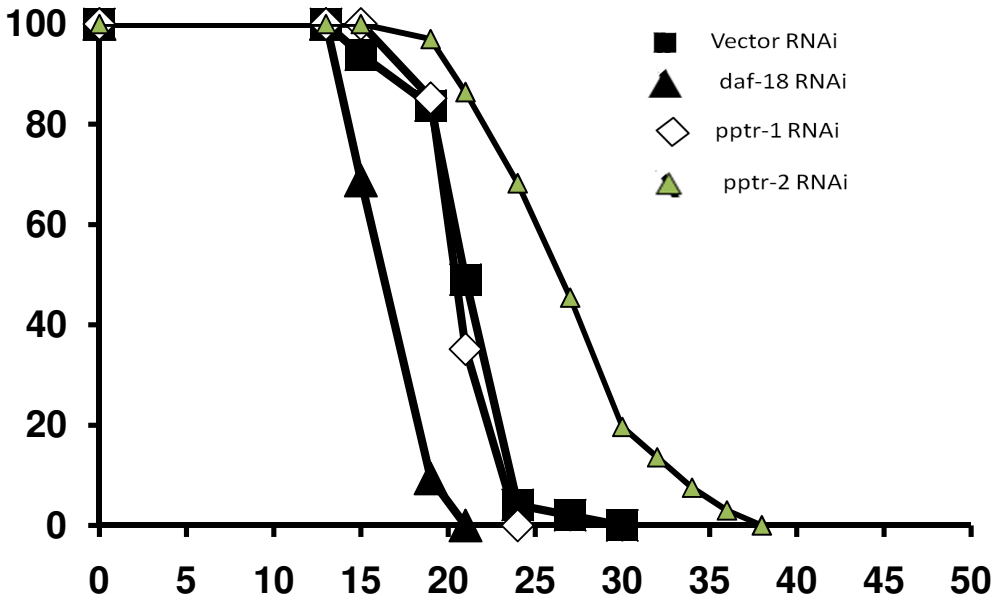
daf-2(e1370);sgk-1(ok538)-dauer assay at 20°C



	dauer	stdev	(n)
vector	84.0	3.2	350
pptr-2	32.2	4.3	393

daf-2(e1370);sgk-1(ok538) dauer formation scored after 10 days at 20°C. *daf-18* and *pptr-1* plates were starved.

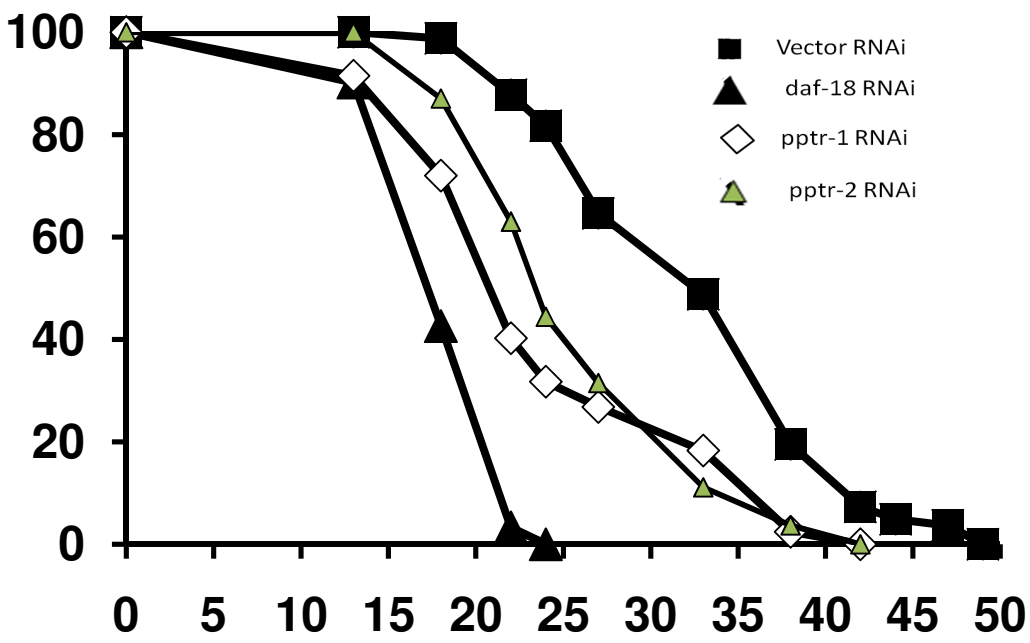
Figure 6



N2 lifespan at 15°C

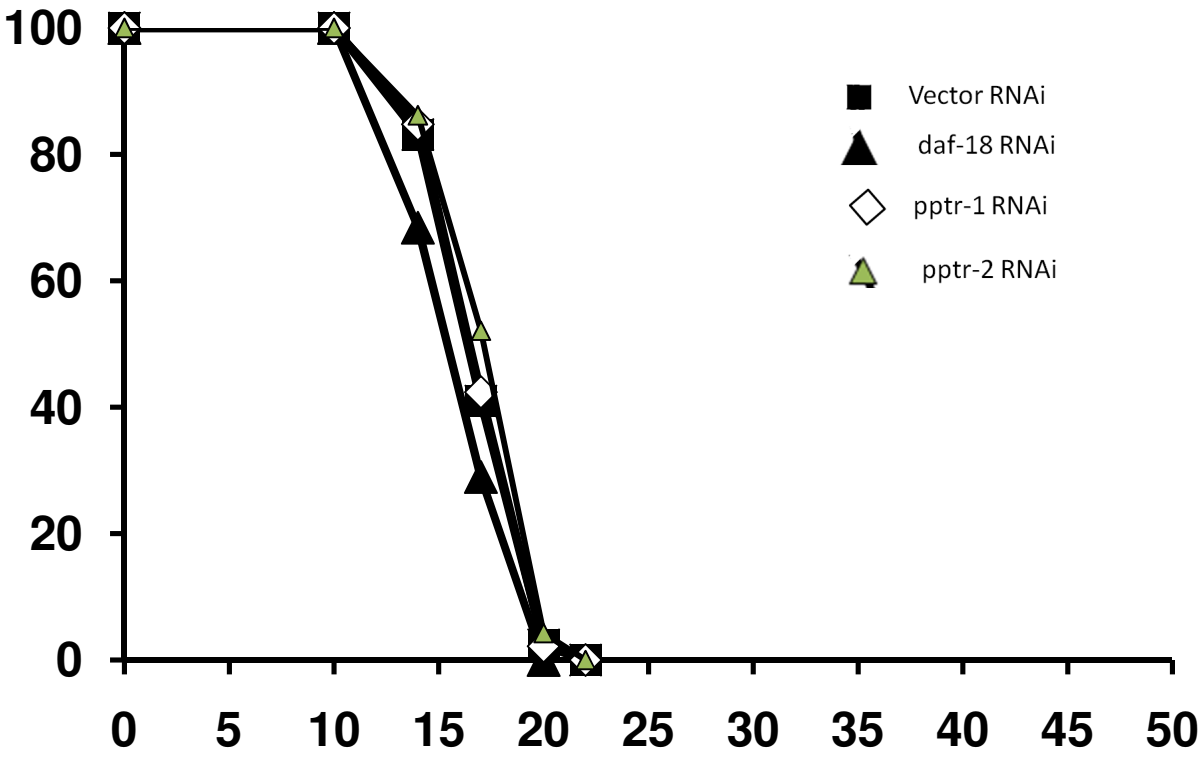
Vector	22.1±0.4
daf-18	17.9±0.3
pptr-1	21.8±0.2
pptr-2	27.8±0.6

Figure 7



daf-2(e1370) lifespan at 15°C	
Vector	33.6±0.9
daf-18	19.3±0.4
pptr-1	24.8±0.9
pptr-2	26.7±0.9

Figure 8



daf-16(mgdf50) lifespan at 15°C

Vector	17.8±0.3
daf-18	16.9±0.3
pptr-1	17.9±0.2
pptr-2	18.2±0.2

Figure 9

A

transcript levels at 15°C (fold change)			
RNAi	*pptr-1*	*pptr-2*	*sod-3*
pptr-1	0.3	1.0	0.6
daf-18	1.3	1.4	0.4
daf-16	1.2	1.5	0.4

B

transcript levels 20°C(fold change)			
RNAi	*pptr-1*	*pptr-2*	*sod-3*
pptr-1	0.5	3.5	0.7
daf-18	1.6	2.2	0.5
daf-16	1.6	1.8	0.2

C

transcript levels compared between 15°C and 20°C(fold change)			
RNAi	*pptr-1*	*pptr-2*	*sod-3*
Vector	0.6	0.6	2.2
pptr-1	1.0	3.8	2.5
daf-18	0.8	0.9	2.6
daf-16	0.8	0.7	1.4

A) *daf-2 (e1370)* worms subjected to RNAi of *pptr-1, daf-18* and *daf-16* grown at 15°C

B) *daf-2 (e1370)* worms subjected to RNAi of *pptr-1*, *daf-18* and *daf-16* grown at 20°C shows upregulation of *pptr-2* by 3.5 fold upon *pptr-1 RNAi*

C) *daf-2 (e1370)* worms were subjected to RNAi of Vector, *pptr-1, daf-18* and *daf-16* and transcript levels of *pptr-1, pptr-2* and *sod-3* compared between 15°C and 20°C

Table 2

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